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Physiological and Biochemical Characterization of a Low Temperature Inducible Protein (BN28) in Acclimating *Brassica napus* cv. Cascade Seedlings.

By

Mitchel D. de Beus ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of

Doctor of Philosophy.

in

Plant Biochemistry

Department of Plant Science

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FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **Physiological and Biochemical Characterization of a Low Temperature Inducible Protein (BN28) in Acclimating *Brassica napus* cv. Cascade Seedlings.** submitted by Mitchel D. de Beus in partial fulfillment of the requirements for the degree of **Doctor of Philosophy** in **Plant Biochemistry.**

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ABSTRACT

Low temperature acclimation induces the accumulation of polypeptides in many plants, including BN28 in the winter cultivar *Brassica napus* cv. Cascade. Although the function of many of these polypeptides remains unknown, two common hypotheses exist in the literature. One suggests that some polypeptides encode antifreeze proteins. The other hypothesis is that cryoprotection, the only other function that directly confers freezing tolerance, can be provided by proteins with no secondary structure. These hypotheses were tested in the present work, which also represents the first attempt to characterize the product of a low temperature-induced gene. A bacterial expression system containing the *bn*28 cDNA was utilized to purify large amounts of bacterial BN28 (rBN28), which was used in biochemical and biophysical studies in addition to being used to produce polyclonal antibodies. Determination of secondary structure revealed that rBN28 lacks an α–helical conformation. As this is required for Type 1 antifreeze protein inhibition of ice crystal formation, it suggests that rBN28 is not an antifreeze protein. A lack of such inhibition activity confirmed that rBN28 does not have antifreeze properties. rBN28 was an effective cryoprotectant, over the physiological range of BN28 concentrations (6 μM to 66 μM), in an *in vitro* assay utilizing lactate dehydrogenase. This report of an amorphous protein having cryoprotective properties provides the first evidence that an unstructured protein is involved in freezing tolerance and can invoke cryoprotection. Further insight into the function of BN28 was gained through *in situ* studies addressing the

temporal and spatial distribution of BN28. High BN28 accumulation was observed in apical meristems and leaf tissue, very little in hypocotyls and petioles and none detected in roots. The highest accumulation occurred in immature leaves, which also attained the highest degree of freezing tolerance. In addition, changes in tolerance were associated with changes in the quantity of BN28. Finally, biochemical analyses were utilized to provide insight into a possible role of BN28 *in situ*. Comparison of rBN28 and BN28 revealed the presence of post-translational modifications to BN28. Analysis indicated alterations in both size and charge. In conclusion, BN28 appears to contribute to freezing tolerance through its cryoprotective capabilities.

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Abbreviations

2-D	2 dimensional
ABA	Abscisic Acid
ADP	Adenosine diphosphate
AFGP	Antifreeze glycoprotein
AFP	Antifreeze protein
AMP	Adenosine monophosphate
Asp	Aspartate
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid
BCIP	5-Bromo-4-chloro-3-indolyl phosphate
bp	Base pair
BSA	Bovine serum albumin
CAP	Cold acclimation protein
cDNA	Complementary deoxyribonucleic acid
COR	Cold regulated
CP_{50}	Cryoprotection value of 50% initial activity
DEAE	Diethylaminoethyl
DEPC	Diethyl pyrocarbonate
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ESR	Electron spin resonance
FAA	Formalin:ethanol;acetic acid
FP	Fluorescence polarization
G6PDH	Glucose-6-Phosphate Dehydrogenase
HEX_{II}	Hexagonal II
IEF	Isoelectric focussing
IgG	Immunoglobulin G
IMP	Inner membrane particle
kDa	Kilodalton
IPTG	Isopropyl β-D-thiogalactopyranoside
KLH	Keyhole limpet hemocyanin
LB	Luria-Bertani
LDH	Lactate dehydrogenase
LEA	Late embryogenesis abundant
LR	London resin
MBP	Maltose binding protein
min.	Minute
mRNA	Messenger ribonucleic acid
N:P:K	Nitrogen: phosphorous: potassium

NADP	Nicotinamide adenine dinucleotide phosphate
NBT	Nitro blue tetrazolium
NMR	Nuclear magnetic resonance
PAGE	Polyacrylamide gel electrophoresis
PAR	Photosynthetically active radiation
PBS	Phosphate buffered saline
PMSF	Phenylmethylsulfonyl fluoride
PSII	Photosystem II
RUBISCO	Ribulose 1,5-bisphosphate carboxylase/oxygenase
SDS	Sodium dodecyl sulphate
TBA	Tertiary butyl alcohol
TBS	Tris buffered saline
TBS-T	Tris buffered saline + Tween
TEM	Transmission electron microscope
Thr	Threonine
TL_{50}	Temperature that causes 50 % ion leakage
Tris-HCl	Tris(hydroxymethyl) methylamine
X-gal	5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside
XR	X ray

Introduction

The distribution of herbaceous plants is regulated by a complex interaction of many environmental factors. In North America and especially Canada, the limiting component that drastically reduces the growing season for many agronomic crops is temperature. A reduction in heat (degree days) retards plant development and crop maturation thereby increasing the time to maturation and increasing the incidence of frost damage. In Alberta, over a ten year period from 1983-1992, frost accounted for 2.5 million dollars per year of damage to canola and 4.4 million dollars per year to wheat, on average (Alberta Crop Insurance Report, 1993).

Studies on the mechanism of freezing damage go back to the 1600's and 1700's, with research being undertaken to understand the causes of frost splitting of trees (Bobart, 1684; Chomel, 1710). Maximov (1912) was the first to suggest that the plasma membrane was the primary site of freezing damage. Since that time one can find thousands of references dealing with the effects of freezing stress on a myriad of physiological and biochemical processes (Scarth, 1936; Scarth and Levitt, 1937; Levitt, 1980; Steponkus, 1984; Huner, 1985; Thomashow, 1990; Guy, 1990; McKersie and Lesham, 1994). Although many hypotheses have been put forth over the years no one hypothesis adequately explains the mechanism of freezing damage and cellular death.

Not all plants succumb to freezing stress at similar temperatures. Many freezing-sensitive plants will die between -2.5°C and -5°C. However, some trees and herbaceous plants have been

shown to withstand -50°C to -60°C, while some tree branches subjected to liquid nitrogen temperature show no damage and flower the following year (Sakai and Larcher, 1987). This is a result of acclimation.

Many biochemical changes occurring in plants during cold acclimation have been studied intensively. These include changes to membrane phospholipids, accumulation of cytoplasmic cryoprotectants (sugars and free amino acids), synthesis of low temperature-functioning isozymes, and the synthesis of other low temperature-specific polypeptides (Sakai and Larcher, 1987; Guy, 1990; Thomashow, 1990; Levitt, 1980; McKersie and Lesham, 1994) Although these biochemical changes have been characterized and the combined effect of these changes are easily seen as increased freezing tolerance, the extent that each change affects plant freezing tolerance is not understood.

Siminovitch and Briggs (1949) correlated the accumulation of soluble protein in cortical bark cells of black locust with increased freezing tolerance. Pomeroy et al. (1970) and Brown (1972) showed that acclimated plant tissue contains 50% to 300% more soluble protein than did the non-acclimated control.

More recently, SDS-PAGE, 2-D gels and *in vitro* and *in vivo* labeling have demonstrated the production of both membrane-bound and soluble polypeptides during low temperature acclimation (Guy and Haskell, 1987; Johnson-Flanagan and Singh, 1987; Faw et al., 1976; McCown et al., 1969; Guy, 1990; Thomashow, 1990).

The advent of molecular biology and differential screening of cDNA libraries has led to the isolation and characterization of a

number of genes that are induced during low temperature treatment. These have been isolated from various plant species, including *Arabidopsis thaliana, Medicago sativa, Triticum aestivum, Spinacia oleracea*, and *Brassica napus* (Guy, 1990).

Workers have begun to study some of the proteins encoded by low-temperature inducible genes and have attempted to determine their function. Despite this, there is little known about any one polypeptide. For example, Hajela et al. (1990) isolated four cold regulated (cor) genes from *Arabidopsis thaliana.* One of these, *cor*15, encodes a protein with cryoprotective properties, suggesting that COR15 may provide protection for enzymes during a freeze stress (Lin and Thomashow 1992a). The CAP85 protein, isolated from spinach, is only responsive to low temperature and water stress (Guy et al., 1985). CAP85 remains soluble during boiling and contains 11 lysine-rich repeats. Both these characteristics are typical of group 2 LEA (late embryogenesis abundant) proteins. *Cor*6.6 encodes an alanine-rich, highly hydrophilic polypeptide that has high homology with *kin*1 of *Arabidopsis thaliana.* The *kin*1 gene encodes a 6.5 kD polypeptide, KIN1 (Kurkela and Franck, 1990). The transcript is induced by low temperature, water stress or ABA, suggesting that the protein may be involved in responses to osmotic stress. KIN1, like COR6.6 (Gilmour et al., 1992)and BN28 (Orr et al., 1992) contains high amounts of alanine and is quite hydrophilic. KIN1 is predicted to have homology with type 1 antifreeze proteins from arctic flounder and, on this basis, has been suggested to be an antifreeze protein (Kurkela and Franck, 1990), inhibiting ice formation through non-covalent interactions with growing ice crystals.

Although various hypotheses have been put forward as to the possible function of low temperature-induced proteins, it is apparent that much of the current knowledge is at best correlative, and often speculative. Thus, the present research was designed to relate structure and function of recombinant BN28, and provide insight into its *in vivo* role. The full length *bn*28 gene was isolated from a cDNA library prepared from cold-acclimated leaves of *Brassica napus* cv Jet Neuf (Orr et al., 1992). The cDNA clone is 0.5 kb in length and encodes a 6.5 kDa peptide (BN28) that has 95% amino acid sequence identity with BRKIN1 (PIR accession#S34660) of *Brassica rapa* and 69%, 66% and 43% homology with COR 6.6, KIN1 and KIN2 of *Arabidopsis thaliana*, respectively.

The objectives of this thesis were to gain insight into the physiological role of BN28 in *Brassica napus* cv. Cascade by studying the accumulation of BN28 during low temperature acclimation conditions in combination with biophysical and biochemical studies. The results from these studies were used to test the following hypotheses:

1) The kin1 gene family, of which *bn*28 is included, encodes antifreeze proteins (AFP) (Kurkela and Franck 1990);

2) Unstructured proteins can protect cytoplasmic proteins from denaturation during dehydrative stress (of which freezing is one);

3) BN28 is involved in the acquisition of freezing tolerance through low temperature acclimation.

In order to address these questions it was required:

a) To produce and purify a significant amount of recombinant BN28 to be used both in the production of polyclonal antibodies and in biochemical and biophysical studies.

b) To study the temporal and spatial distribution of BN28 in *Brassica napus* cv. Cascade seedlings during acclimation through the use of western blotting, immunohistochemistry, and immunolocalization.

c) To determine secondary structure of rBN28 and compare this with the predicted secondary structure for BN28.

d) To determine if rBN28 has recrystallization inhibition activity similar to AFPs.

e) To determine if rBN28 can protect a freeze-labile enzyme (LDH) from freeze denaturation through an *in vitro* cryoprotection assay.

f) To determine the physiological range of concentrations for BN28 in acclimated leaf tissue during a freeze stress, and to use that information to determine the cryoprotective properties of rBN28 in an *in vitro* cryoprotection assay.

g) To begin to compare and contrast the biochemistry of BN28 and the bacterially expressed BN28 (rBN28) to determine and identify possible post-translational modifications to the protein.

Literature Review

A) Formation of Ice Crystals

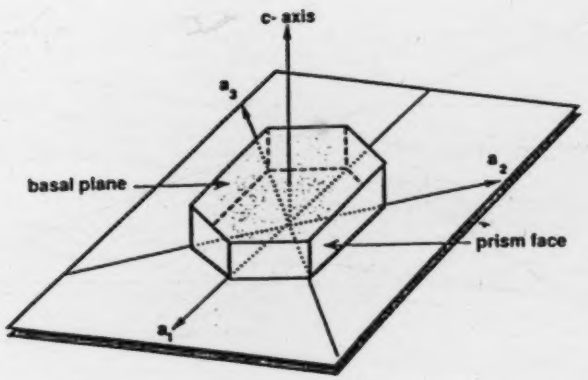
When pure water is cooled at atmospheric pressure it remains in a liquid state well below 0°C. This is referred to as supercooling (Hew and Yang, 1992). Under these conditions water forms high density clusters of molecules that exhibit an arrangement similar to ice crystals. These clusters or ice nuclei are unstable and dissipate rapidly. As the temperature is decreased these nuclei become more stable and increase in size. Once they reach a critical size to mass ratio, spontaneous ice crystallization occurs. This is referred to as homogeneous ice nucleation, which occurs at the supercooling threshold temperature (-40°C for pure water).

In impure water, molecules arrange themselves around foreign particles and form pseudo ice nuclei. Upon further cooling the critical size to mass ratio is prematurely met and heterogeneous ice nucleation occurs. The threshold temperature for heterogeneous ice nucleation is usually between -2°C and -15°C (Hew and Yang, 1992).

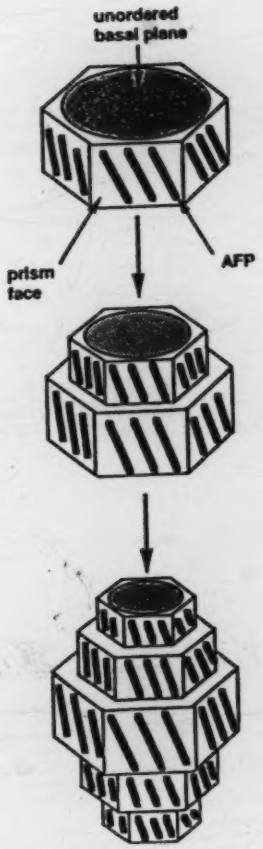
Ice crystals below freezing temperatures are always found in the hexagonal form containing a basal plane, 6 prism faces and a c-axis (Fig. 1). Normal ice growth occurs along the 6 prism faces forming long needle-like ice that can cause severe tissue damage.

Fig. 1. The mode of function for a Type 1 antifreeze protein binding to ice nuclei and altering the morphology of ice crystal formation. A) A schematic representation of an hexagonal ice crystal. Normal ice growth occurs on each prism face. B) A schematic representation of the interaction of AFP interaction with ice (Taken from Hew and Yang, 1992).

A



B



1. AFP binds preferentially to prism faces through dipolar and hydrogen bond interactions.

2. This results in ordering of water-dipoles that lie within the field of AFP helix-dipoles (unshaded area).

3. Ice still grows on unordered basal plane (shaded area).

4. AFP binds to new ice fronts.

5. Continued ice growth on basal plane, and continued binding of AFP to prism faces results in bipyramidal ice crystals.

B) Freezing Stress in Plant Tissue

The formation of ice crystals is the primary stress induced by freezing temperatures in plant tissue. The level of strain that is observed is a direct response to the inherent potential of the plant to freeze. If tissue is rapidly cooled at rates greater than 5°C per hour, the formation of intracellular ice is probable (Morren, 1838; Goppert, 1883; Molisch, 1897; Siminovitch and Scarth, 1938). The ice crystals are long and needle-like and cause disruption as they elongate; this is lethal. Mazur (1960) showed that rapid cooling of yeast led to less than 1% survival versus 50% survival when cooling was slower. Asahina (1956) confirmed that in higher plants, cell death during rapid cooling was directly caused by intracellular freezing. Conversely, tissues cooled at rates under 5°C per hour undergo extracellular ice formation, which may not lead to cell disruption during freezing (Caspary, 1854).

During slow cooling of leaf tissue, ice crystals first occur in large xylem vessels (Asahina, 1956; Kitaura, 1967). Several points within each vessel act as nucleation points. Just prior to nucleation both the extracellular spaces and adjacent cell cytoplasms are at temperature and vapor pressure equilibrium. Seeding within the vessels causes the formation of a small crystal of ice, which causes a decrease in vapor pressure in the extracellular space. Under these conditions, water flows from the adjacent cytoplasms through the plasma membrane into the extracellular space to maintain vapor pressure equilibrium. If freezing is gradual, this cycle will continue and lead to an increased amount of extracellular ice, causing cellular plasmorhysis.

The limiting factor in extracellular ice formation is the rate of water diffusion from the cytoplasm through the plasma membrane.

i) Freezing-Induced Membrane Damage

Maximov (1912) hypothesized that the plasma membrane was the primary site of freezing damage. This began decades of research on the biochemical and physical properties of membranes and the effects of freezing on membrane structure (Harvey, 1918; Gordon-Kamm and Steponkus, 1984; Steponkus, 1984; Uemura and Yoshida, 1984; Johnson-Flanagan and Singh, 1986; Lynch and Steponkus, 1987; Mohapatra et al., 1988; Uemura and Steponkus, 1989, 1994).

To simulate freezing stress, protoplasts isolated from non-acclimated rye tissues were subjected to hypertonic salt solutions (Steponkus, 1984; Dowgert and Steponkus, 1984). At moderate levels of salt, the dehydrative stress caused protoplast plasma membranes to become appressed forming endocytotic vesicles that were apparently lost to the cytoplasm. Upon deplasmolysis the protoplasts could not re-expand fully, and therefore underwent expansion-induced lysis. When protoplasts from acclimated tissue were subjected to similar levels of osmotic stress, exocytotic extrusions of the plasma membrane were observed upon plasmolysis. These extrusions did not result in a decrease in plasma membrane area. Therefore, upon deplasmolysis, expansion-induced lysis was prevented. However, if the level of salt stress was increased, protoplasts from acclimated tissue eventually lost osmotic responsiveness.

Cell walls, which protoplasts lack, are integral in the dehydration response. Johnson-Flanagan and Singh (1986) studied the role of cell walls on plasma membrane alterations in *Medicago sativa* and *Brassica napus* suspension cultured cells and in isolated mesophyll cells from *Secale cereale* cv Puma. During plasmolysis, cells from acclimated tissue sequestered plasma membrane as a large number of exocytotic membrane strands between the protoplast and the cell wall. Upon deplasmolysis these strands were incorporated back into the plasma membrane. Plasmolyzed non-acclimated cells formed many thin exocytotic membrane strands. On deplasmolysis, this membrane material was lost to the expanding protoplast and led to expansion-induced lysis.

Singh et al. (1987) addressed ultrastructural alterations in winter rye mesophyll cells and *Brassica napus* cultured suspension cells during dehydrative stress. It was shown that at lethal temperatures (-5°C) multilamellar vesicles formed both inside the vacuole and on the cell surface of non-acclimated cells of both rye and *Brassica napus*. At -5°C these vesicles were quite loosely packed but became highly appressed at -10°C. When the same non-acclimated cells were plasmolysed with a balanced salt solution, to induce a dehydrative stress, multilamellar vesicles and intravacuolar tonoplast vesicles were absent at the low salt concentration and appeared as the salt concentration increased. Therefore, there was a continuum of responses to an increase in dehydrative stress. They concluded that both expansion-induced lysis and loss of osmotic responsiveness resulted from the formation of multilamellar vesicles in non-acclimated *Brassica napus* cells and rye tissue.

Exposure of cellular membranes to freezing conditions (-10°C) also alters the organization of membrane components, leading to the formation of regions devoid of proteinaceous intramembrane particles (IMP) (Pearce and Willison, 1985a,b; Gordon-Kamm and Steponkus, 1984; Webb and Steponkus, 1993) and loss of osmotic responsiveness. Freeze-etched control and acclimated leaf tissue revealed a uniform distribution of proteinaceous intramembrane particles (IMP) in plasma membranes not subjected to a damaging freezing stress. However, when non-acclimated tissue was subjected to a level of stress that could induce freeze damage, the formation of IMP-free areas was observed. As plants underwent acclimation, the extent of IMP-free areas, formed at a given temperature, decreased. It was hypothesized that these IMP-free domains are regions of membrane that undergo a phase change from liquid-crystalline to a more rigid gel phase. Although the mechanism is unknown, it is believed that this physical change to the membrane lipids leads directly to a redistribution of IMP outside of these rigid domains.

Freeze-fracture analysis of membranes, frozen to -10°C, led to the identification of both Hex_{II} and fracture-jump lesions within the plasma membrane (Gordon-Kamm and Steponkus, 1984; Webb and Steponkus, 1992, 1993; Webb et al., 1994). Gordon-Kamm and Steponkus (1984) subjected isolated protoplasts from non-acclimated rye leaves to freezing stress. Protoplasts from non-acclimated tissue held at 0°C showed a uniform distribution of IMP throughout the plasma membrane. Tissue that was cooled and held at -10°C for 15 min showed lateral phase separations and extensive IMP-free domains. When non-acclimated rye protoplasts were subjected to a

hypertonic solution to mimic freezing-induced dehydration. membranes showed similar perturbations (Gordon-Kamm and Steponkus, 1984). Therefore, it was concluded that the observed membrane alterations are a result of freeze-induced dehydration and not other temperature effects. Webb and Steponkus (1993) showed that protoplasts from acclimated rye leaves formed little Hex_{II} phase at temperatures as low as -35°C but did form significant amounts of fracture-jump lesions within the plasma membrane. It was hypothesized that fracture-jump lesion structures occur through either freeze-induced interdigitation of lipids in the plasma membrane or through freeze-induced fusion of the plasma membrane with closely appressed lamellae from the endomembranes (Webb and Steponkus, 1993; Webb et al., 1994).

The effects of low temperature inducible proteins on expansion-induced lysis and loss of osmotic responsiveness were recently addressed in *in vitro* assays utilizing liposomes (Uemura et al., 1996; Webb et al., 1996). To determine possible roles for two low- temperature induced polypeptides (COR6.6 and COR15) in membrane stabilization, Uemura et al. (1996) studied the effects of these peptides on freeze-induced fusion and leakage by placing them inside liposomes. The addition of COR6.6 or COR15 to liposomes from acclimated *Arabidopsis* membrane lipids showed a reduction in the amount of freeze-induced fusion but no change in leakage. When COR6.6 or COR15 were tested in liposomes from non-acclimated *Arabidopsis*, similar results were obtained. Therefore, it was suggested that the reduction of fusion in liposomes from acclimated and non-acclimated tissue was believed to be a consequence of an

effect of COR peptides with either ice formation or ice liposome interactions (Uemura et al., 1996).

The effects of COR peptides on dehydration-induced phase transitions of liposomes subjected to osmotic stress were also addressed (Webb et al., 1996). It was shown that the incorporation of COR6.6 or COR15 inside liposomes did not prevent or reduce the incidence of phase transitions from lamellar to HEX_{II}. These results together with those of Uemura et al. (1996) strongly suggested that these COR peptides did not have a direct cryoprotective effect on liposomes.

ii) Secondary Freezing Damage

The literature shows that plasmolysis-induced increases in the concentration of cytoplasmic solutes leads to alterations in enzymatic (Markert, 1963; Levitt, 1966; LaMotte et al., 1969; Lund et al., 1969; Tong and Pincock 1969) and non-enzymatic reactions (Fennema et al., 1966; Grant and Alburn, 1967; Grant, 1969). The rate of reaction for the oxidation of ascorbic acid was 3 times higher in tissue frozen at -11°C than in tissue at 1°C (Grant, 1969). Also, the acid-catalyzed hydrolysis of acetic anhydride was 15 times higher at -10°C than that detected at 5°C (Grant and Alburn, 1967). Similarly, the hydroxylation of glutamine occurred 300 times faster at -18°C than that at 0°C (Grant, 1969).

Reaction rates of enzymes are also affected by the dehydrative component of freezing stress. Tong and Pincock (1969) observed that invertase activity in yeast, frozen at -4°C, was 1.6 times higher than if it was kept in the supercooled state at -4°C. It was suggested

that the increase in activity was in response to an increase in the enzyme-substrate affinity (Tong and Pincock, 1969).

Exposure to freezing stress has also been shown to inactivate enzymes (Markert, 1963; Levitt, 1966; LaMotte et al., 1969; Lund et al., 1969; Tong and Pincock 1969). Lund et al. (1969) observed that the reaction rate for the enzyme-catalyzed hydrolysis of sucrose by invertase slowly decreased between 12°C and 0°C. However, when exposed to sub-zero temperatures, the activity rapidly decreased. When the temperature reached -22°C, less than 0.01% of the original invertase activity occurred (Lund et al., 1969). The rapid reduction in activity was not believed to be brought about by a change in enzyme-substrate affinity, as observed by Tong and Pincock (1969), but rather, caused by the dissociation of the enzyme (Lund et al., 1969).

While addressing the role of pectin esterase in abscission zones of leaves and adjacent stem tissues of *Coleus blumei* and *Phaseolus vulgarus*, it was observed that a 3 hour freeze at -15°C reduced the activity of pectin esterase to 38 % of the original non-stressed activity (LaMotte et al., 1969). This suggested that the enzyme was freeze-labile.

The property of freeze-lability was also observed for a number of other enzymes. These include lactate dehydrogenase in animals (Markert, 1963), triosphosphate dehydrogenase in plants (Chilson et al., 1965) glutamate dehydrogenase in plants (Chilson et al., 1965), catalase in animal (Shikama, 1963). Therefore, inactivation of enzymes through freezing is common in both plant and animal tissues.

The freeze-lability of lactate dehydrogenase has led to its usage in cryoprotective studies. Lin and Thomashow (1992a) and Kazuoka and Oeda (1994) have shown that peptides induced during low temperature acclimation in plants can prevent the freeze-induced denaturation of LDH in an *in vitro* cryoprotection assay.

C) Low Temperature Exposure

Exposure to low non-freezing conditions induces a wide range of changes in plants. These include alterations in plant anatomy (Huner et al., 1981; Griffith and Brown, 1982; Huner, 1985; Griffith et al., 1985), plant physiology (Dexter, 1933; Scarth and Levitt, 1937; Levitt 1980; Hurry and Huner, 1992; Öquist et al., 1993; Hurry et al., 1994, 1995 a,b) and biochemistry (Huner and MacDowall, 1976, a,b, 1979 a,b; Holiday et al., 1992; Hurry et al, 1995 a,b). These changes are involved in at least 1 of four processes: a) the acquisition of freezing tolerance (low temperature acclimation); b) the metabolic adjustment to growth at low temperature; c) initial low temperature stress; d) vernalization.

i) Low Temperature Acclimation

Stone et al. (1993) suggested that all plants have an inherent level of freezing tolerance. In addition, many temperate perennial, annual and biennial plants have a second separate level of freezing tolerance that is induced in response to shorter day length and/or low non-freezing temperatures. For example, Fowler et al. (1983) showed that the LT_{50} of field grown winter wheat was -3°C in September, -22°C in December and -3°C by May.

Examples exist in the literature of changes brought about by low temperature exposure that are directly related to the acquisition of freezing tolerance (Guy and Haskell 1987; Charest and Phan, 1991; Öquist et al., 1993). During acclimation of winter wheat, a positive correlation was found between freezing tolerance of the various cultivars and the capacity to maintain the plastoquinone pool in the oxidized state (Öquist et al., 1993). A similar correlation was observed between freezing tolerance of the plant and its light-saturated rate of photosynthesis following hardening (Öquist et al., 1993). It was suggested that the capacity to change the light-saturated rate of photosynthesis and the ability to keep plastoquinone fully oxidized represented a specific photosynthetic adaptation involved in the acquisition of freezing tolerance.

Charest and Phan (1991) studied the kinetic properties of proline dehydrogenase, glutamate dehydrogenase, glutamine synthase and ornithine transaminase in proline metabolism during cold acclimation. Analysis of these proteins in both winter and spring wheat showed that the catalytic efficiency of proline dehydrogenase did not change in the winter cultivar during acclimation. However, the other 3 enzymes showed a slight increase in V_{max} and a significant decrease in Km values during acclimation, that was not observed in the spring cultivar. These changes were proposed to enhance the formation of proline.

Guy and Haskell (1987) showed, through *in vivo* labeling of spinach seedling leaves, that the peptides CAPs 160, 117 and 85 are synthesized in response to low temperature exposure and no longer synthesized during deacclimation. Thus, the synthesis and lack of

synthesis of CAPs 160, 117 and 85 were highly correlated with the acquisition and loss of freezing tolerance. Whether BN28 plays a similar role in acclimation remains to be seen.

ii) Metabolic Adjustments for Low Temperature Growth

No evidence exists for the direct involvement of low temperature-inducible proteins in the metabolic adjustment for low temperature growth. However, experimental evidence exists for low temperature changes to metabolism (Holiday, 1992; Hurry 1994, 1995a). Analysis of spinach plants exposed to low temperatures showed that there was an increase in the total activity of stromal fructose 1,6-bisphosphatase, sedoheptulose 1,7-bisphosphatase and cytosolic fructose 1,6-bisphosphatase (Holaday et al., 1992) as well as an increase in sucrose phosphate synthase. When spinach leaf disks were transferred from 27°C to 10°C, stromal fructose 1,6-bisphosphatase and NADP-malate dehydrogenase reached maximum activities in 8 minutes, while RUBISCO reached 99% maximum activity in 15 minutes of low temperature exposure. Holaday et al. (1992) suggested that low temperature exposure leads to an increase in the maximum activities of enzymes.

CO_2 saturation rates of photosynthesis at 5°C were 68% greater in acclimated tissue than in non-acclimated tissue. This increase in photosynthetic capacity in cold hardened plants correlates with the increase in RUBISCO activity and the large increase in the substrate pools of phosphorylated intermediates (Hurry et al., 1994). It was suggested that the strong recovery of photosynthesis in cold hardened winter rye indicates a build-up of photosynthetic enzymes

and intermediates allowing them to maximize sugar production for the winter (Hurry et al., 1994, 1995a).

Rye plants exposed to low temperature acclimating conditions expressed a modified RUBISCO enzyme with an altered isoelectric point (Huner and Macdowall, 1976a,b). This implies that low temperature induced alteration in RUBISCO may support enhanced activity at sub-optimal temperatures (Huner and Macdowall. 1976a,b; 1979a,b).

iii) Initial Low Temperature Stress

Evidence exists in the literature for low temperature-induced changes that are strictly low temperature effects and have no role in acclimation, metabolic adjustments or vernalization. Studies of changes in fatty acid composition in 4 wheat cultivars with contrasting hardiness demonstrated a uniform degree of stimulation in linolenic acid biosynthesis resulting in the uniform unsaturation of membrane lipids (de la Roche et al., 1975). These results suggested that unsaturation was not directly related to the different freezing tolerances of cultivars but probably was a general response to low temperature exposure. de la Roche (1979) confirmed these results through the use of inhibitors of linolenic acid synthesis. Seedlings of winter wheat were acclimated in the presence of various inhibitors. Although the amount of linolenic acid was reduced by approximately 50% over the non-treated controls, the survival of the seedlings was unaffected. These results confirmed that enrichment in linolenic acid is not a prerequisite for low temperature acclimation and that it is simply a low temperature response.

Sarhan and Chevrier (1985) studied the regulation of RNA synthesis by DNA-dependent RNA polymerases and RNases during low temperature growth in winter and spring wheat. They showed that total RNA polymerase activities were significantly higher in chromatin isolated from low temperature grown winter wheat as compared to that obtained from spring wheat grown under low temperatures. This correlated with an increase in protein and RNA during hardening. However, the chromatin-associated RNase activity decreased in both winter and spring wheat during acclimation, indicating that changes in chromatin RNase activities are not related to the cold hardening process but rather are an effect of low temperature.

iv) Vernalization

The requirements for vernalization (lower temperatures and shorter day lengths) are similar to the requirements for low temperature acclimation (Murphy and Scarth, 1994). Therefore, the changes that are observed during low temperature exposure become difficult to categorize. Laroche et al. (1992) identified a low temperature inducible change that was strictly involved in vernalization. They studied ribosomal DNA (rDNA) in spring and winter types of *Brassica napus* during low temperature exposure. An increase in rDNA copy number was observed in the winter cultivar but not in the spring cultivar. Further study of rDNA copy number was undertaken on more crucifer species capable of developing significant freezing tolerance, but differing in vernalization requirements. A relationship was observed between

increasing rDNA copy number and vernalization. Therefore, it was suggested that this increase in rDNA is a vernalization effect (Laroche et al., 1992). The role of low temperature-inducible peptides in vernalization has not yet been addressed.

V) Plant Anatomy

Growth rates of plants grown at cold hardening temperatures are markedly reduced (Huner, 1985). Puma rye grown at non-acclimating conditions obtained maximum vegetative growth by 28 days, while those seedlings grown at cold-hardening temperatures reached maximum vegetative growth by 56 days (Huner, 1985). Growth at low temperatures had no effect on leaf numbers, but did alter leaf dry weight, fresh weight, and area (Krol and Huner, 1984). Examination of winter rye *Secale cereale* grown and developed at low temperatures revealed enlarged mesophyll cells which led to a 50% increase in leaf thickening. Low temperature exposure caused a reduction in stomatal frequency. The abaxial surface of unhardened leaf tissue contained 12.8 stomates per unit area, which decreased to 5.4 in hardened leaf. Similarely, the adaxial surface decreased from 7.9 stomates to 3.9 in hardened leaf (Huner et al., 1981). Asymmetric thickening of the mestome sheath and epidermal cells was also observed. It was hypothesised that thickening occured through enhanced suberization (Griffith and Brown, 1982). It has been suggested that the accumulation of lipid polymers may physically compartmentalize water in the leaf to prevent ice propagation from xylem to mesophyll tissue (Griffith et al., 1985).

D) Protein Changes Induced by Low Temperature

The literature shows that low temperature exposure can alter the cellular composition of some proteins (Siminovitch and Briggs, 1949; Siminovitch et al., 1968; Cloutier, 1983; Robertson et al., 1988). For example, Siminovitch and Briggs (1949) showed that the accumulation of soluble protein in cortical bark cells of black locust was correlated with an increase in freezing tolerance. It was also shown that acclimated wheat and black locust have a 50% and 300% increase in soluble protein as compared with their non-acclimated controls (Siminovitch et al., 1968; Trunova, 1982). Cloutier (1983) observed differences in electrophoretic patterns of soluble proteins in 7 cultivars of winter wheat and rye following acclimation.

Robertson et al., (1988) while studying bromegrass (*Bromus inermus* Leyss.) suspension cultured cells, were able to correlate changes in soluble proteins with increased freezing tolerance. They showed there was an accumulation of several polypeptides during acclimation. These included a 20 kDa, 165 kDa, 190 kDa and a 200 kDa polypeptide. It was also observed that accumulation of a 22 kDa peptide was decreased.

i) Protein Distribution

Very little is known about the distribution of low temperature inducible protein. Guy et al. (1988) showed several acclimation-specific proteins in leaf and stem tissue of spinach that were not

found in the roots. Similarly, Perras and Sarhan (1989) identified several proteins that occur only in leaves.

Cellular distribution of low temperature inducible proteins varies as much as tissue distribution. Subcellular distribution of low temperature inducible proteins revealed that some are in the chloroplast (Lin and Thomashow, 1992b) while others in the nucleus (Asghar et al., 1994; Houde et al., 1995), endoplasmic reticulum (Neven et al., 1993) and the cytosol (Neven et al., 1993; Kazuoka and Oeda, 1994; Houde et al., 1995). To date, only 1 other study has been undertaken utilizing immunolocalization techniques to examine organ/tissue and sub-cellular accumulation of a low temperature-induced protein *in situ*. Houde et al. (1995) noted the accumulation of freezing-tolerance-associated proteins (WCS) in the cytoplasm and nucleoplasm of acclimated wheat crown tissues. Although interesting, the relationship between plant developmental stage and organ development and the accumulation of low temperature inducible proteins has yet to be addressed.

ii) *In vivo* Labeling

In vivo labeling of plant material with ^{35}S methionine shows that low temperature exposure alters the synthesis of various polypeptides within tissues. Observed changes include the degradation of some proteins along with the synthesis of other acclimation-specific peptides (Cracker et al., 1969; Faw et al., 1976; Huner and Macdowall, 1976a; McCown et al., 1968; Kurkela et al., 1988; Gilmour et al., 1988; Robertson et al., 1988; Perras and Sarhan, 1989; Guy and Haskell, 1987; Guy, 1990; Thomashow, 1990). In the

few cases where a relationship between freezing tolerance and the synthesis of new polypeptides is found, workers have been unable to demonstrate anything more than a correlation.

iii) *In vitro* Translation

Weiser (1970) was the first to suggest that plants may contain a "unique" set of genes that are induced only under low temperature acclimating conditions. Utilizing *in vitro* translation of poly (A+) mRNA isolated from control and cold acclimated tissues, researchers have shown that differences in gene expression exist between acclimated and non-acclimated tissue (Guy et al., 1988; Kurkela et al., 1988; Gilmour et al., 1988; Mohapatra et al., 1987a,b). Gilmour et al. (1988) showed that mRNA specific to acclimation encode a 160 kDa polypeptide and 4 other polypeptides in the range of 47 kDa in *Arabidopsis thaliana*. Kurkela et al. (1988) also identified 3 low temperature inducible polypeptides in the *in vitro* translation products from acclimated *Arabidopsis* tissue (150 kDa, 45 kDa and 24 kDa). Although this technique clearly showed that low temperature acclimation induces expression of a gene set, it becomes difficult to relate the expression of a unique mRNA to an increase in freezing tolerance.

iv) Isolation of cDNAs

The advent of cDNA technology has allowed the isolation of a large number of low temperature inducible plant genes. These include the *cor* and *kin* genes of *Arabidopsis* (Gilmour et al., 1988; Lång et al., 1989; Kurkela and Franck, 1990; Wilhelm and

Thomashow, 1993), *cas* genes in alfalfa (Mohapatra et al., 1987a,b), *wcs* genes in wheat (Sarhan and Perras, 1987; Perras and Sarhan, 1989), *cor* genes in spinach (Guy and Haskell, 1987; Guy et al., 1988) and *bn* genes in *Brassica* (Orr et al., 1992; Weretilnyk et al., 1993). Utilizing differential screening of cDNA libraries, constructed from acclimated *Arabidopsis thaliana* leaf tissue, Hajela et al. (1990), isolated 4 cDNAs (pHH7.2, pHH28, pHH29, pHH67). Northern blot analyses revealed that the transcripts increased markedly from 1 to 4 hours of exposure, reached a maximum by 12 hours and remained high as long as the plants were maintained at low temperature.

Weretilnyk et al. (1993) utilized differential screening of cDNA libraries constructed from *Brassica napus* cv Jet Neuf leaf tissue. This led to the isolation of several cDNA clones (pBN115, pBN19 and pBN26). Northern blot analysis of *bn*115 revealed that it was induced within 1 day of low temperature exposure. Although drought stress led to a slight increase in transcript level at room temperature, heat shock and ABA had no effect on the level of message. Northern blot analysis with cDNA clones pBN19 and pBN26 showed cross-hybidization with pBN115 suggesting that pBN115 may be part of a gene family.

Several cDNA clones were isolated in response to low temperature in wheat (*Triticum aestivum*) (Houde et al., 1992), including *wcs*120. Northern blot analysis revealed that *wcs*120 message was induced within 1 day in response to cold. Antibodies produced to WCS protein were utilized to screen cDNA expression libraries. The antibody cross reacted with two other proteins leading to the isolation of two more genes (*wcs*200 and *wcs*66). Analysis of

the 3 genes revealed that they contain 6 copies of a 23 amino acid consensus sequence and 11 copies of a 14 amino acid consensus sequence.

Chauvin et al. (1993) isolated the cDNA clone *Wcs*19 from wheat. Northern blot analysis reveals that the *Wcs* message accumulates within 1 day in cultivars with high freezing tolerance (LT_{50} -19°C) when first exposed to acclimating conditions and remains high under low temperature conditions. In a less tolerant cultivar (LT_{50} -8°C), the mRNA accumulates within 1 day, but declines over time even though the plant is under acclimating conditions.

A great deal of information has been obtained through these and other studies. However, they have not provided any information on the role of these proteins. Inferences of possible protein function are being made from results of sequence comparisons and Northern blotting (Kurkela and Franck, 1990; Gilmour et al., 1992). These are strictly correlative at best and speculative at worst.

v) Gene Induction Through Alternate Stresses

Understanding which stresses induce the accumulation of a protein, combined with an understanding of common strains found between stresses may help to provide insight into the physiological role of the protein. Dallaire et al. (1994) addressed the role of ABA and low temperature in the acquisition of freezing tolerance. The application of ABA to winter and spring cultivars of wheat leaves for 5 days alone or following 7 days of low temperature acclimation produced maximum freezing tolerances (LT_{50}) of -8°C and -6°C.

Conversely, the maximum levels of freezing tolerance (LT_{50}) obtained after 40 days acclimation was -17°C and -9°C. These differences in maximum freezing tolerances between the two treatments suggested that ABA and low temperature acclimation induce freezing tolerance via distinct regulatory pathways in wheat (Dallaire et al., 1994).

Examples of common regulatory pathways between stresses exist in literature. It has been shown that pre-exposure of wheat and rye to limited desiccation for 1 day can induce freezing tolerance equal to 4 weeks acclimation (Chen et al., 1975; Siminovitch and Cloutier, 1982; Cloutier and Andrews, 1984). *Kin*1 and *kin*2 of *Arabidopsis* are both inducible by the phytohormone ABA but only *kin*2 is induced by salinity and drought (Kurkela and Borg-Franck, 1992). COR genes of *Arabidopsis* are inducible by low temperature, ABA and drought, but not heat shock (Hajela et al., 1990).

vi) Low Temperature Inducible Proteins

The following is a brief description of selected low temperature-induced genes and their gene products. Currently, none of the products of these genes have been biochemically and biophysically characterized.

a) HSC70 and BN59

Anderson et al. (1994) isolated the gene encoding the endoplasmic reticulum (ER) luminal HSC70 from a cDNA library of poly(A+) RNA from 2-day cold acclimated spinach seedling leaves. Northern blot analysis showed that this gene was upregulated following 1 day of acclimation and remained highly expressed

through 1 day of deacclimation. Since HSP70s are considered to be involved in the folding of proteins, their induction by low temperature supports the concept that they may be important for maintaining homeostasis and proper protein function during cold acclimation and during a freeze stress.

The only other low temperature inducible gene for which a function is known is *pbn59* which encodes a 70 kDa subunit of the vacuolar H^+ ATPase in *Brassica napus* (Orr et al., 1995). *pbn59* transcript levels became detectable after 1 day low temperature and continued to increase over 14 days exposure. *bn*59 transcript was also induced by drought and ABA to levels approaching that induced by low temperature. Western blotting of endomembrane fractions of *Brassica napus* leaves during acclimation revealed that BN59 began to accumulate to detectable levels by 1 week of low temperature and reached maximum levels by 4 weeks. This supports a possible role for BN59 in maintaining ATP production through acclimation and during a freeze stress.

b) WCS200

WCS200 is a 200 kDa peptide that is induced specifically by low temperature in wheat (*Triticum aestivum*). It is an hydrophilic polypeptide that remains in solution following boiling. It contains 11.4% glycine, 13.3% threonine and 22% alanine (Ouellet et al., 1993). Tryptic digests showed that the protein contains at least 3 copies of a lysine-rich repeat and at least 5 copies of a glycine-rich repeat. The peptide is synthesized in greater amounts in the leaves and shoot apex than in the roots. WCS200 is the largest of the low

temperature inducible peptides isolated to date. Immunological analysis showed that WCS200 is a member of a protein family that includes several peptides ranging from 40 kDa to 200 kDa (Houde et al., 1992). One protein, encoded by *wcs*120, contains 6 copies of the lysine-rich repeat and 11 copies of the glycine-rich repeat. Houde et al. (1995) determined that in acclimated wheat leaves the total cellular concentration of WCS proteins was 1.34 µM. They also showed that WSC120 protected LDH from freeze-denaturation in an *in vitro* assay within this concentration range.

c) COR47, COR15 and BN115

Genes encoding a number of cold regulated proteins (COR) have been isolated from *Arabidopsis thaliana*. Amino acid sequence analysis of COR47 showed that it has high homology with late embryogenesis abundant (LEA) proteins (Gilmour et al., 1992), suggesting that it may play a role in water regulation during freeze-induced dehydration.

COR15 is a hydrophilic peptide that was tested for cryoprotective properties in an *in vitro* assay measuring residual LDH activity following a freeze-thaw stress (Lin and Thomashow, 1992a). COR15 is synthesized as a 15 kDa peptide that is processed to 9.4 kDa by cleavage of the transit peptide and is found in the stromal fraction of chloroplasts from acclimated tissue (Gilmour et al., 1996). Two dimensional gels showed that COR15 and recombinant COR15 have similar mobilities, suggesting that the native protein is not post-translationally modified.

White et al. (1994) isolated the *cor*15 homologue in *Brassica napus*, BN115. Analysis of upstream regions identified two 8-bp elements which are identical to the positive regulatory regions of the promoter of the *cor15* gene and to a 5-bp core sequence in the low temperature and dehydration responsive elements in promoter regions of several cold responsive *Arabidopsis thaliana* genes.

d) CAP85

The cDNA for an 85 kDa protein (CAP85) was isolated from a cDNA library constructed from poly(A)$^+$ RNA from 2 day acclimated spinach leaves (Neven et al., 1993). The protein was induced in response to both low temperature and water stress. Western blot analysis revealed that CAP85 was present in all aerial tissues as well as the root tissue. Sub-cellular determination showed that it is predominantly found in the cytosol. Amino acid analysis of CAP85 revealed that the protein contains an 11 amino acid, lysine-rich repeat characteristic of a Group 2 LEA. Therefore, they suggested that CAP85 may be involved in water binding and in minimizing freeze-induced cellular dehydration.

e) KIN1 and KIN2

Very little is known about these proteins. Genes encoding KIN1 and KIN2 were isolated from a cDNA library constructed from acclimated leaf tissue from *Arabidopsis thaliana*. Northern blotting suggests that *kin*1 mRNA increased 20-fold during cold treatment of leaf tissue. *kin*1 is induced by ABA while *kin*2 is also induced by salinity and drought (Kurkela and Borg-Franck, 1992). *kin*1 and *kin*2

contain 3 exons separated by introns. The coding region is 95 % homologous to that of *kin*1, while the homologies between the introns are 80% and 65%, respectively. Both sequences are 66 amino acids long, corresponding to 6.5 kDa polypeptides and contain high amounts of alanine, glycine and lysine. Sequence comparisons showed that KIN1 and KIN2 have similarities to fish antifreeze proteins (AFP). On this basis, KIN proteins and their homologues were predicted to function as AFPs (Kurkela and Franck, 1990).

f) COR6.6 and COR15

Sequence analysis of COR6.6 showed that the peptide is hydrophilic and alanine-rich (Gilmour et al., 1992). It has 96% amino acid homology with KIN1 and is a cytoplasmic 6.6 kDa protein. The recombinant form of COR6.6 migrated as a single band whereas COR6.6 exists as multiple forms, of which most appear to be more acidic than the recombinant form (Gilmour et al. 1996).

Recombinant COR 6.6 and COR15 were tested for their ability to prevent freeze-induced fusion and leakage of liposomes, and to prevent or reduce dehydration-induced phase transitions in *in vitro* assays. As noted in the section "Freezing-induced membrane damage", the researchers were unable to ascribe a function to either protein.

g)BN28

Currently, very little is known about BN28 and its possible role in low temperature acclimation. The cDNA encoding BN28 was isolated through differential screening of a λgt10 library constructed

from *Brassica napus* cv Jet Neuf mRNA (Orr et al., 1992). It was determined, from the deduced amino acid composition, that BN28 is composed of 65 residues of which 34% are alanine and glycine. The peptide lacks tyrosine, tryptophan, cysteine, and proline. The polypeptide has a predicted molecular weight of 6.5 kDa and isoelectric point of 10 (Orr et al., 1992). BN28 has 95% amino acid sequence homology with BRKIN1 (PIR accession #S34660) of *Brassica rapa*, 69% with COR 6.6 and 66% and 43% amino acid sequence homology with KIN1 and KIN2 of *Arabidopsis thaliana*, respectively. Further comparisons between BN28, COR6.6 and KIN 1 are shown in Table 1. Transcript levels of *BN*28 reach a maximum within 1 day acclimation and remain highly expressed while the plant is maintained at low temperatures. When the plant is returned to non-acclimating conditions the mRNA disappears within 12 hours.

E) Low Temperature Inducible Proteins with Known Function

While much of the emphasis on identification of low temperature-induced proteins has been on altered gene expression, a small group of workers have taken an alternative approach. By searching for proteins with a given function, antifreeze proteins, thermal hysteresis proteins and cryoprotective proteins have been identified.

i) Antifreeze and Thermal Hysteresis Proteins

The perception of low temperatures and shorter day length, leading to an increase in freezing tolerance, is not restricted to the plant kingdom. Many organisms have unique mechanisms to

minimize freezing damage induced by extracellular ice formation. One such approach in fish and insects involves the annual synthesis of antifreeze proteins and thermal hysteresis factors that inhibit or alter pattern of ice crystal growth and increase freezing tolerance (DeVries, 1971; DeVries and Lin, 1977; Wen and Laursen, 1992; Hew and Yang, 1992).

a) Antifreeze Proteins of Fish

Experimental evidence suggest that polar fish accumulate a variety of proteins that are involved in supercooling and osmoregulation (Scholander et al., 1957). Scholander and Maggert (1971) reported the presence of macromolecular compounds with thermal hysteresis properties in arctic sculpins. DeVries confirmed the presence of these macromolecules in Antarctic fish and identified them as antifreeze proteins (DeVries, 1971). Biochemical characterization of these peptides shows that they can be divided into two major classes: the antifreeze glycoproteins (AFGP) and antifreeze proteins (AFP). AFGP range in size from 2.6 to 34 kDa and make up 3.5% of fish blood serum. Most freezing tolerant fish show 1 of 8 variants all containing an $(Ala\text{-}Thr\text{-}Ala)_n$ repeat with the carbohydrate moiety attached to the Thr (Hew and Yang, 1992).

AFP are divided into 3 classes. Type I AFP are between 3.3 and 4.5 kDa with an 11 amino acid repeat $(Ala\text{-}Ala\text{-}Ala\text{-}Ala\text{-}Ala\text{-}Ala\text{-}Leu\text{-}Thr\text{-}Ala\text{-}Ala\text{-}Asp)_n$. They contain high amounts of alanine and have an α-helical structure. Type II AFP are slightly larger, ranging from 11.3 to 24 kDa and are cysteine rich. They contain disulfide bonds and have a substantial amount of ß-sheet. Very little is

known about type III AFP. The average size is 6 kDa and their amino acid composition and secondary structure varies (Hew and Yang, 1992).

b) Antifreeze Protein Mode of Action

Research has shown that type 1 AFP function through the adsorption to the surface of ice nuclei (Raymond and DeVries, 1972). Studies of the freezing profiles of pure water solutions containing NaCl and AFP showed that NaCl broadened the transition temperature of freezing water while AFP maintained a sharp transition. This indicated that the antifreeze proteins were incorporated into the ice. Utilizing light microscopy and polarizing light it was shown that AFP was adsorbed to the surface of the ice crystals (Raymond and DeVries, 1972). DeVries and Lin (1977) attributed the binding of α-helical antifreeze proteins (Type 1 AFP) on the prism faces of ice to hydrogen bonding between oxygen atoms on the ice with the polar groups on the α-helical AFP. It was shown that the intermolecular distance between Thr and Asp or Asn residues in type 1 AFP was 0.45 nm. This corresponded exactly to the 0.45 nm separating adjacent oxygen atoms in ice crystals, supporting the hypothesis for hydrogen bonding.

As shown in Fig. 1, the AFP preferentially binds to the 6 prism faces (Hew and Yang, 1992), resulting in ordering of the water molecules that lie around the AFP (unshaded area). This prevents ice crystal growth in the a-axes, however, ice still grows on the un-ordered water found in the basal plane (shaded area). The AFP then bind to the new ice fronts. This sequence continues resulting in the

formation of bipyramidal ice crystals. The alteration in ice crystal morphology leads to freezing point depression of the solution without affecting the melting point. This is referred to as thermal hysteresis (DeVries, 1971). Simultaneously, the AFP inhibits the formation of long needle-like ice crystals that are destructive to surrounding tissue, replacing them with bipyramidal ice crystals that are much less destructive (DeVries and Lin, 1977; Hew and Yang, 1992). This prevents tissue damage and increases freezing tolerance.

c) Thermal Hysteresis and Ice Nucleators of Insects

It has been shown that unique thermal hysteresis factors accumulate in insects subjected to low temperatures (Ramsay 1964; Duman, 1977; Duman et al., 1991). Ramsay (1964) first reported the presence of unique particles in the body fluid of the beetle *Tenebrio molitor*. Duman (1977) later identified these particles and others as thermal hysteresis factors. Since then, thermal hysteresis proteins have been found in many insects including most freeze-avoiding types (Zettel, 1975; Patterson and Duman, 1978; Horwath and Duman, 1984; Duman et al., 1991). Analysis of isolated thermal hysteresis proteins revealed that many peptides contain 12%-28% cysteine and form disulfide bridges (Schneppenheim and Theede, 1980; Patterson and Duman, 1982). Further analysis suggested that the thermal hysteresis factors of insects are not glycoproteins (Zachariassen, 1985).

d) Plant Thermal Hysteresis Proteins

Recent identification of thermal hysteresis factors in plants has revealed common mechanisms of freezing tolerance between animals, insects and plants (Griffith et al., 1992; Urrutia et al., 1992; Duman, 1994; Hon et al., 1994). Urrutia et al. (1992) found 16 plant species showing some level of thermal hysteresis. Exudate from stems of bittersweet nightshade, *Solanum dulcamara*, collected in the fall showed thermal hysteresis levels of 0.45°C. This level was reduced to 0.28°C by March and had completely disappeared by May, suggesting that the accumulation of thermal hysteresis proteins is cued and controlled by shorter day length and reduced temperature. The fall samples produced fewer and finer ice crystals, as compared with the spring sample, correlating with the expected effect of thermal hysteresis proteins. Treatment with dithiothreitol led to loss of thermal hysteresis activity suggesting that thermal hysteresis proteins contain disulfide bonds.

There are differences in thermal hysteresis protein levels throughout the plant. Comparisons of thermal hysteresis activity of exudate from various organs of *Solanum dulcamara* collected in the fall showed activity in the primary stem of 0.45°C as compared with 0.21°C for the secondary stem and 0.22°C for the leaf (Urrutia et al., 1992). Thermal hysteresis proteins in plants account for an increase in freezing tolerance of only 0.5°C, while the overall freezing tolerance of plants must approach -20°C to -40°C in winter. Although thermal hysteresis proteins accounts for a small change in tolerance,

freezing tolerance is a multigenic trait with each gene product contributing a small part to the overall tolerance of the plant.

Duman (1994) purified and characterized a thermal hysteresis protein from leaf tissue of *Solanum dulcamara*. The purification procedure isolated a 67 kDa polypeptide, which is considerably larger than some animal thermal hysteresis proteins. Amino acid analysis showed that the peptide is composed of 23.7 mol % glycine. Treatment of purified thermal hysteresis protein with periodate or borate caused inactivation indicating the presence of a carbohydrate moiety. More specific treatment with ß-galactosidase also inactivated the protein. This confirmed the presence of galactose and indicated that one component of plant thermal hysteresis factors is a glycopeptide.

Griffith et al. (1992) looked for apoplastic antifreeze proteins in acclimated winter rye leaves. Proteins isolated from the apoplastic space have a concentration dependent effect on ice crystal morphology. However, at physiological concentrations thermal hysteresis activity was minimal (0.33°C at 60 mg/ml AFP). One must question the physiological significance of such a small increase in tolerance in a plant that can acclimate to a very low temperature.

Further characterization of the apoplastic exudate revealed the presence of seven major peptides ranging from 11 to 36 kDa (Hon et al., 1994). Assessment of antifreeze activity of the individually purified peptides was carried out by observing their ability to modify the growth habits of ice crystals. It was shown that 5 of the 7 peptides had significant levels of antifreeze activity, but the others had very little effect on ice crystal formation. Addition of

dithiothreitol to the proteins caused changes in the electrophoretic mobility in 5 of the 7, suggesting the presence of disulfide bonds. However, unlike other proteins, antifreeze activity was not lost upon this treatment. Amino acid analysis of the 5 peptides shows similar profiles for all. They appear to be rich in aspartate, glutamate, serine, threonine, glycine and alanine (Hon et al., 1994).

e) Plant Transformations

Experimental evidence suggested that the presence of fish AFP or synthesized forms of AFP vacuum infiltrated or transformed into plant tissue conferrs thermal hysteresis activity (Cutler et al., 1989; McKown and Warren, 1991; Hightower et al., 1991; Kenward et al., 1993). Cutler et al. (1989) vacuum infiltrated the winter flounder antifreeze protein (1 mg/ml) into leaves of potato (*Solanum tuberosum*), *Brassica napus* and *Arabidopsis thaliana*. They observed a 1.8°C decrease in freezing point over that in the control tissue. They also measured the amount of freezable water in the infiltrated tissue and found a reduction in the presence of the antifreeze protein. Thirdly, they observed that the infiltrated protein decreased the rate of ice crystal formation. On this basis, it suggested that antifreeze proteins have multiple roles in plant tissue acting as anti-nucleators, cryoprotective compounds and as antifreeze proteins (Cutler et al., 1989).

McKown and Warren (1991) transformed yeast with different chimeric genes consisting of 2 to 5 copies of the characteristic 11-amino acid repeat from winter flounder antifreeze protein and tested the transgenics for enhanced ice recrystallization inhibition. They

observed that the construct containing 5 copies of the repeat had the greatest effect on decreasing recrystallization and increasing the survival of yeast after rapid freezing (95°C/min to -196°C) and moderate rates of warming (26°C to 64°C/min). It was estimated that the chimeric protein accounted for 0.04% of the total yeast cellular protein.

The first published attempt to transform plants with the full length cDNA encoding a type I antifreeze protein from winter flounder was carried out by Kenward et al. (1993). The cDNA was expressed using the cauliflower mosaic virus 35S promoter. Initial studies showed accumulation of antifreeze protein mRNA in transgenic plants. However, Western blots failed to show detectable levels of antifreeze proteins in transgenic plants grown under control conditions. Upon further analysis they discovered that the transgenic plants did accumulate antifreeze proteins when the plants were exposed to low temperature acclimating conditions. Since the promoter was not known to be temperature-sensitive, post-translational regulation was suggested. Evidence to support this comes from a study of circular dichroism of the type I antifreeze protein showing that the proportion of α-helix decreases from 85% at -1°C, to 47% at 25 °C and 0% at 70°C (Ananthanarayanan and Hew, 1977). Kenward et al. (1993) suggested that the transgenic antifreeze protein, with less α-helix at 25°C, was more susceptible to proteolysis. Therefore, they hypothesized that protein accumulation may have been dependent on secondary structure, although no direct evidence was presented.

Hightower et al. (1991) produced transgenic tobacco and tomato plants transformed with a chimeric gene containing 5 copies of the 11-amino acid alanine-rich repeat from a fish antifreeze protein and a transit peptide targeting the protein to the extracellular space. Both transgenic tobacco and tomato plants expressed high mRNA levels of the chimeric gene. Only samples extracted from transformed tomato plants contained the protein and displayed recrystallization inhibition activity. The concentration of the chimeric peptide was approximately 100 ng or 0.1% of the total soluble leaf protein.

f) Antifreeze Proteins with Multiple Functions

The variability of size and composition of all antifreeze proteins and thermal hysteresis factors suggests that these proteins could have a second, yet undetermined function within the plant. Sequence analysis showed that the antifreeze peptides from rye tissue are similar to 3 classes of pathogenesis-related proteins (Hon et al., 1995). These include endochitinases, endo-ß-1,3-glucanases and thaumatin-like proteins. The preliminary classification of the proteins was confirmed by observing enzyme activities and testing for immuno-cross-reactivity.

Chitinases and glucanases purified from acclimated rye leaves have strong antifreeze activity (Hon et al., 1995). However, similar purified enzymes from tobacco had no antifreeze activity, suggesting that the bi-functional property of the rye proteins is specific for rye plants. Tronsmo (1984, 1985, 1993) showed that acclimation of freeze-tolerant grasses also induces resistance to fungal diseases

such as leaf spot, snow molds, rusts and powdery mildew. It has been suggested that the anti-pathogen and antifreeze properties of these peptides can confer simultaneous resistance to both biotic and abiotic stresses (Hon et al., 1995). However, this is a generalization. Chitinases and glucanases from low temperature grown tobacco have no antifreeze activity (Hon et al., 1995).

ii) Cryoprotectants

Organisms accumulate low molecular weight soluble compounds during acclimation that enhance freezing tolerance (Withers and King, 1979; Levitt, 1980; Santarius and Giersh, 1983, 1984; Nadeau et al., 1987; Koster and Lynch, 1992; Kishitani et al., 1994). It was shown that these cryoprotectants including carbohydrates such as sucrose, raffinose and sorbitol, a number of polyamines such as putrescine, spermidine, spermine and cadaverine, as well as the amino acid, proline and ammonia compounds such as glycine betaine (Levitt, 1980).

There are two functions of cryoprotectants: protection of biomembranes; and prevention of protein denaturation. Santarious (1982) hypothesized that compounds conferring freeze-thaw protection on isolated chloroplast membranes did so by reducing the solute permeability of membranes during freezing and by increasing membrane expandibility during thawing. From this, a cryoprotection assay was developed based on maintaining chloroplast integrity. This assay has been used to evaluate cryoprotective proteins. For example, Hincha et al. (1989) isolated protein fractions from acclimated *Brassica oleracea* cv Grufiwi leaves that protected

chloroplasts. Analysis of the fractions revealed that 1 contained a 28.5 kDa peptide. Hincha et al. (1990) also isolated other protein fractions from acclimated cabbage and spinach that showed cryoprotective properties. Recently, Sieg et al. (1996) continued this work and purified a 7 kDa glycoprotein with cryoprotective activity.

Carpenter and Crowe (1988) hypothesized that protection of proteins occurs through preferential exclusion of solutes. During preferential exclusion the cryoprotectant added does not bind to the protein, leading to an unfavorable increase in free energy in solution. This increase in free energy is thought to cause the preferential hydration and stabilization of proteins (Carpenter and Crowe, 1988). To address this hypothesis an *in vitro* assay was established to assess the effectiveness of a "cryoprotectant" in preventing the freeze denaturation of a freeze labile enzyme LDH (Santarious, 1982).

Utilizing this assay, some low temperature inducible proteins were shown to exhibit this type of cryoprotective property. Lin and Thomashow (1992a) showed that COR15 has cryoprotective levels 10^6 times more effective than sucrose and was 100 to 1000 times more effective than BSA. Similarly Kazuoka and Oeda (1994) showed that COR85, isolated from cold acclimated spinach, had a higher level of cryoprotection than that of 9 proteins tested, including BSA, myoglobin, cytochrome-c, lyzozyme and RNase A.

Recently, Houde et al. (1995) showed that WSC120 isolated from acclimated wheat tissue exhibits cryoprotective behavior at physiological concentrations (0.58µM). WSC120 retained 50 % LDH activity (CP_{50}) at 10 µg/ml (or 0.2 µM), which is slightly better than BSA (CP_{50} 12.5 µg/ml).

Materials and Methods

A) *E. coli* Transformation with pMAL-C.

Bacterial transformation and BN28 induction were carried out as described in the procedures from Protein Fusion and Purification Systems (New England Bio Labs, Beverly, U.S.A). The *Brassica napus* cDNA clone encoding *bn*28 had been previously cloned into a fusion protein expression vector pMAL-c. Plasmid (2 ng) modified by Boothe et al. (1995) was mixed with 25 µl competent D5Hα LacZα complementary *E. coli*, incubated on ice for 5 min. and heated to 42°C for 2 min. LB media (100 µl) was added, incubated at 37°C for 20 min, then plated on LB with ampicillin (100 µg/ml) and incubated overnight at 37°C. Colonies were transferred to a LB-amp (100 µg/ml) plate with 80 µg/ml X-gal and 0.1 mM IPTG. Again the plates were incubated overnight at 37°C. White colonies representing transformed colonies were selected and maintained on LB-amp plates for BN28 production.

B) rBN28 Purification

LB-amp (100 µg/ml) (500 ml) was inoculated with 5 ml of overnight culture containing transformed *E. coli*. The culture was grown at 37°C to a density of $2x10^8$ cells per ml (A_{600}=0.5). IPTG was added to the culture to a final concentration of 0.3 mM. After incubation for 3 h at 37°C, to induce synthesis of the maltose binding protein (MBP-BN28) fusion protein, the cells were centrifuged at 4000 x g for 20 min and resuspended in 5 ml 25 mM Tris-HCl, pH 8.0. Cells were frozen overnight at -20°C and thawed in cold water

(4°C). The cells were lysed by sonicating, with a Sonic 300 dismembrator (Artek Systems Corp., Farmingdale NY, U.S.A.), 3 times for 30 sec each at 35% full power with a micro tip attachment. The cellular debris was removed by centrifuging at 100,000 x g for 1 h. The crude protein (CP) was desalted twice by loading 1 ml fractions onto a PD-10 column (Pharmacia, Uppsala, Sweden) packed with G-25 coarse resin (Pharmacia, Uppsala, Sweden) pre-swollen in 25 mM Tris-HCl, pH 8.0, and centrifuging in a clinical centrifuge for 10 min at 3000 x g. Total desalted CP (5 ml) was mixed in a similar column packed with 5 ml DEAE cellulose (Pharmacia, Uppsala, Sweden) pre-swollen in 25 mM Tris-HCl, pH 8.0. After incubation at 4°C for 15 min, with frequent agitating, the column was again centrifuged for 10 min at 3000 x g to remove unbound bacterial proteins. The DEAE cellulose containing the bound MBP-BN28 was washed several times in the column, centrifuging repeatedly, with a total of 50 ml 25 mM Tris-HCl, pH 8.0. Factor Xa protease (25 µl) was diluted with 6.0 ml of 25mM Tris-HCl, pH 8.0. This was then combined with the DEAE resin and incubated overnight at 4°C with continuous agitation to allow cleavage of the fusion protein. The column was centrifuged for 10 min at 3000 x g to collect cleaved BN28 from the DEAE cellulose containing the bound MBP portion of the fusion protein.

C) Protein Determination

Quantification of leaf and *E. coli* protein was carried out using the Bicinchoninic acid (BCA) assay (Pierce, Rockford, U.S.A.) (Smith et al., 1985). Extracted SDS-soluble leaf protein was diluted 1:100 with water. Twenty-five µl was added to 500 µl working reagent.

incubated at 60°C for 30 min and the absorbance was then measured spectrophotometrically at 562 nm. Protein concentrations were determined from a standard curve using known amounts of bovine serum albumin (BSA).

Purified rBN28 was quantified by diluting it 1:10 with water and measuring its absorbance at 205 nm. The concentration of C in (mg/ml) was calculated from Beer's Law:

$$A_{205}=E_{205}lC \quad \text{where } E_{205}=31$$

$$l=1 \text{ cm}$$

D) Polyacrylamide Gel Electrophoresis (SDS-PAGE) of Polypeptides

SDS-PAGE was carried out using a Tris-Tricine discontinuous gel system as outlined by Schagger and von Jagow (1987). Acrylamide gels (8.5 cm x 7.5 cm x1.5 mm) were used with a 4% stacking gel and a 16% separating gel. Protein samples (60 µg) were made to 40 µl with SDS-PAGE sample buffer (50 mM Tris-HCl, pH 8.0, 2% SDS, 5% 2-mercaptoethanol, 10% glycerol, 0.025% bromophenol blue), boiled for 5 min and loaded on the gel. Gels were run at 35 mA per gel for 3 h or until the dye front reached 1 cm from the bottom of the gel.

E) Coomassie Blue Staining of SDS-PAGE Gels

SDS-PAGE gels were stained in staining solution (50% methanol, 0.05% Coomassie brilliant blue R-250 (Pierce, Rockford, U.S.A), 10% acetic acid and 40% water) overnight. They were then destained in destain solution (50% methanol, 10% acetic acid and 40% water) for

several hours or until the background was reduced and the protein bands became visible.

F) Silver Staining of SDS-PAGE Gels

Silver staining was carried out as outlined by Morrissey (1983). Proteins were fixed in the polyacrylamide gel by 30 min rinses in 50% ethanol, 10% acetic acid, followed by 5% ethanol, 5% acetic acid and finally 10% glutaraldehyde in water. After rinsing well with water (3 x 30 minutes) the gels were soaked in 5 mg/1000 ml dithiothreitol (DTT) for 30 min. The DTT solution was removed, and then without rinsing, the gel was soaked for 30 min in 0.1% silver nitrate. The gels were developed by rinsing twice briefly with water then twice briefly in developer (0.5% formaldehyde (BRL) in 3% Na_2CO_3). The reaction was inhibited by the addition of 5% 2.3 M citric acid.

G) Anti-BN28 Polyclonal Antibody Production

To enhance the antigenicity of BN28, it was conjugated to Keyhole Limpet Hemocyanin (KLH), a large carrier molecule (Sigma, ST. Louis, U.S.A). Equal masses (300 µg) each of recombinant BN28 and KLH were combined in PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HP0_4 and 1.4 mM KH_2P0_4, pH, 7.3) and crosslinked with an equal volume of 0.2% glutaraldehyde in PBS for 12 hours at room temperature (Harlow and Lane, 1988). The samples were centrifuged on a PD-10 column (Pharmacia, Uppsala, Sweden) equilibrated with PBS to remove the glutaraldehyde. Conjugated protein was mixed with an equal volume Freund's complete adjuvant

at which time antigens were administered through subcutaneous injections. The initial sample (800 µl) was injected at 4 sites (200 µl per site) on the rabbit. Second and third injections were administered 14 days apart. Two weeks after each boost 15 ml of blood was taken from each of two rabbits. Blood was kept overnight at 4°C to promote clotting at which time the clot was removed and the serum was centrifuged at 13000 x g for 20 min to remove particulate matter (Harlow and Lane, 1988).

H) Anti-BN28 Purification

Antibody purification was carried out by a modification of the immunoblotting technique (Harlow and Lane, 1988). Purified rBN28 polypeptide (100 µg per well) was run on a five well preparative SDS gel and electroblotted onto nitrocellulose as outlined in section K. The blot was blocked overnight with TBS-T with 5% BSA, rinsed briefly with TBS-T, incubated for 2 h with polyclonal anti-BN28 polyclonal antibodies diluted 1:200 with TBS-T and 0.3% BSA and rinsed twice for 10 min with TBS-T. One strip from the outer two wells was cut from the blot to determine the location of rBN28. These strips were incubated with goat anti-rabbit IgG secondary antibodies and colour development was carried out as outlined in section K. Once BN28 was located on the blot, the nitrocellulose containing BN28 was placed in 5.0 ml 100 mM glycine, pH 2.5 and vortexed frequently for 10 min to remove bound anti-BN28 antibodies. The nitrocellulose was removed and 0.5 ml 1M Tris-HCl, pH 8.0 was added. The purified antibodies were made up to 0.3% BSA and centrifuged at 13500 x g for 2 min to remove insoluble

material. The purified antibodies were then used in all immunocytochemistry and immunolocalization experiments.

I) Plant Material

Brassica napus cv. Cascade plants were grown from seed in a peat-lite Cornell mix under a 18/6 h day/night photoperiod at 400 µmol $m^{-2}s^{-1}$ photosynthetically active radiation (PAR) at 20/15°C. Seedlings were watered daily and fertilized weekly with 20:20:20 (N:P:K). Once the first, fourth or seventh true leaf had expanded to two centimeters in diameter (young, intermediate or older seedlings, respectively) the plants were acclimated under a 16/8 h photoperiod at 4°C under reduced light intensity (200 µmol m^{-2} sec^{-1} PAR) for up to 42 days.

J) Leaf Protein Extraction

Approximately 100 mg of leaf tissue was frozen with liquid nitrogen and ground with a mortar and pestle. Leaf protein extraction buffer (0.5 ml) consisting of 50 mM Tris-HCl buffer, pH 8.0, 1 mM PMSF and 2% SDS, was added while grinding continued and the tissue began to thaw. Upon thawing the slurry was centrifuged at 13500 x g, for 5 min in a centrifuge. The supernatant was removed, half was stored at -80°C for protein determination, and the other half was diluted 1:1 with SDS-PAGE sample buffer (50 mM Tris-HCl, pH 8.0, 2% SDS, 5% ß-mercaptoethanol, 10% glycerol, and 0.05% bromophenol blue) boiled at 100°C for 5 min and stored at -80°C.

For native and urea gels, protein was extracted as for SDS-

soluble proteins with slight modifications. For both, the grinding buffer consisted of 50 mM Tris-HCl buffer, pH 8.0 and 1 mM PMSF. After centrifugation, the proteins were diluted in modified loading gel buffers as outlined below, and isolated proteins were not boiled.

K) Immunoblotting

Western blotting was carried out as described by Hames and Rickwood (1990). Gels and membranes were soaked for 5 min in Dunn's buffer (10 mM $NaHCO_3$, 3mM Na_2CO_3 and 20% methanol, pH 9.9) before proteins were electroblotted at 300 mA for 2 h onto 0.2 μm nitrocellulose membranes (Schleicher and Schuell, Keene, U.S.A.) (Dunn, 1986). Membranes were blocked overnight at room temperature in TBS-T (10mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM Na_2EDTA, 0.1% Tween 20 and 0.01% Thimersal) with 5% BSA, then briefly rinsed with TBS-T and incubated with rabbit anti-BN28 polyclonal antibodies, diluted 1:200 with TBS-T and 0.03% BSA, for 2.5 h at room temperature. Membranes were washed 3 times for 10 min with TBS-T and then incubated for 2 h with goat anti-rabbit IgG antibodies conjugated with alkaline phosphatase (Sigma, St. Louis, U.S.A.) diluted 1:3000 with TBS-T. Blots were rinsed twice for 10 min with TBS-T and twice for 10 min with TBS (10 mM Tris-HCl, pH 7.4 and 150 mM NaCl). Colour development was initiated by incubating the blot with 0.1 M $NaHCO_3$ pH 9.8, 1 mM $MgCl_2$, 300 mg/ml NBT and 150 mg/ml BCIP. The colour reaction was stopped by several rinses with distilled water and a final soak with TBS and 20 mM EDTA.

L) Cytoplasmic Water Determination in Frozen Leaf Tissue

Unfrozen water determinations were carried out as described by Burke et al. (1974) and Gusta et al. (1975) with slight modifications. Fully acclimated (6 weeks) 4 leaf stage seedlings were watered to saturation 16 h prior to experimentation to ensure full turgor. One half of leaf 4 (1 cm x 2 cm) was weighed and placed in a 5 mm NMR tube (Norell Inc, Landisville, U.S.A). The second half of leaf 4 was weighed for fresh weight determination then ground in liquid nitrogen for total SDS-soluble protein determination. The NMR tubes and leaf tissue were equilibrated at +2.5°C for 2 h at which time the unfrozen water curve was measured with a Bruker 80 MHZ NMR(Bruker, Washington, U.S.A) at +2.5°C. The integrated area under the curve represented 100% unfrozen water ($A_{2.5}$). The samples were returned to a programmable freezer and the temperature was lowered to -2.5°C over two hours. After equilibrating at -2.5°C for one hour the samples were nucleated with ice crystals from a glass rod dipped in liquid nitrogen. The temperature was held for 11-13 h then lowered at -2.5°C/hour until the desired temperature was reached. Samples were removed at -5, -10, -15, -17.5°C and again the water curves were measured on the NMR equilibrated to sample temperature. The integrated area under the curve represented the amount of unfrozen water at a given temperature (A_T). Afterwards, dry weights were determined for each sample by heating at 80°C for 3 days or until the dry weights for the samples remained constant. From these measures the total grams of water per gram dry weight was calculated (L_{10}).

Gusta et al. (1975) showed that the total grams of unfrozen water per gram dry weight (L_T) could be calculated from the following equation.

$$L_T=L_{10}(A_T/A_{2.5})(T/T_{2.5})$$

To determine the concentration of BN28 in cells during a freeze stress three parameters were needed: 1) Quantity of BN28 in an acclimated leaf. Q= ng BN28/50 µg total SDS soluble protein. 2) The amount of unfrozen water at different temperatures in acclimated leaf tissue L_T= g H_2O/g dry weight. 3) Total SDS soluble protein per gram dry weight of acclimated tissue. P= µg protein/ g dry weight.
To determine BN28 concentration [BN28] = Q x P / L_T

From previous studies Q= 0.5 ng BN28/g total protein.
(Boothe et al., 1995)

P= 419360 µg protein/g dry weight.
L_T= varies from 5.2 to 0.47.

M) Recrystallization Inhibition Activity

Recrystallization inhibition activity of rBN28 was assessed as outlined by Hightower et al. (1991). Droplets (5µl) containing 0-1500 µg/ml of rBN28 in 25 mM Tris-HCl pH 8.0 were dropped on a polished metal plate at -70°C to form small ice nuclei. The frozen drops were transferred to a cryostage at -7°C. Following 1-2 h equilibration, ice crystal structures were monitored. Ice crystal structures were compared with Tris buffer containing no rBN28.

N) Cryoprotection Assays

Cryoprotection assays were performed as described by Lin and Thomashow (1992a) with slight modifications. rBN28, proline, glycine, BSA and sucrose were frozen in 1.5 ml microfuge tubes at various concentrations in 50 mM Tris-HCl, pH 8.0, containing 1.25 µg/ml type III lactate dehydrogenase (LDH), from bovine heart (Sigma), at -20°C for 18 h. Upon thawing on ice, 20 µl of each concentration was added to 1 ml of assay mixture (80 mM Tris-HCl, pH 7.5, 100 mM KCl, 2 mM pyruvic acid (Sigma), 0.3 mM NADH (Sigma). LDH activity was determined by measuring the change in absorbance at 340 nm. CP_{50} values were defined as the amount of cryoprotectant required to maintain 50% LDH activity following an overnight freeze at -17°C.

As controls of this assay, LDH activity was determined after an overnight incubation in the absence of rBN28 at 4°C. Also, the effect of adding rBN28 to an aqueous solution of LDH before and following a freeze-thaw stress was also addressed. rBN28 was added at the physiological range of BN28 concentrations (40 µg/ml to 400 µg/ml) following an overnight freeze at -17°C. Following incubation at room temperature for 30 min LDH activity was again measured at 340 nm.

Cryoprotection with rBN28 was also assessed in an *in vitro* assay observing the activity of the freeze labile enzyme glucose-6-phosphate dehydrogenase (G6PDH) (Tamiya et al., 1985). G6PDH activity was assayed from soluble proteins isolated from leaf 4 of non-acclimated seedlings at the four leaf stage. Leaf tissue (200 mg)

was disrupted in 1 ml enzyme extraction buffer (50 mM potassium phosphate buffer pH 8.0 with 2mM EDTA at 4°C) with two 10 sec pulses from a polytron homogenizer. The homogenates were centrifuged 2 times for 5 min each at 13000 x g. The final supernatant was used in the cryoprotection assay. In a final volume of 1 ml, assay mixture (100 mM Tris-HCl, 3 mM glucose-6-phosphate, 2 mM EDTA (Sigma), 0.2 mM $NADP^+$ (Sigma) pH 8.0 at 25 °C) and supernatant were combined. G6PDH activity was measured by following the reduction of $NADP^+$ at 340 nm (Fickenscher and Scheibe, 1986). The amount of homogenate added to the reaction mixture was adjusted to obtain a initial rate of $NADP^+$ reduction of 0.05 µM per minute at 340 nm. Once this volume was determined, samples of homogenate were made up to the physiological range of BN28 (40 µg/ml or 400 µg/ml) with rBN28 and initial G6PDH activities were determined for each sample. Following an overnight freeze at -17 °C residual G6PDH activity in the thawed samples was measured by making the same volume of sample up to 1 ml with assay mixture and following the change in absorbance at 340 nm. The addition of excess glucose-6-phosphate to the reaction mixture insured the G6PDH-mediated reduction of $NADP^+$ was rate limiting while other dehydrogenases would have very little effect because of a lack of substrate (Farr et al.,1994). To test this the homogenate was added to assay mixture lacking glucose-6-phosphate to observe background $NADP^+$ reduction. Again, as controls of this assay, G6PDH activity was determined after an overnight incubation in the absence of rBN28 at 4°C. Also, the effect of adding rBN28 to an aqueous solution containing G6PDH before and following a freeze-thaw stress

was addressed.

O) Native and Urea Polyacrylamide Gel Electrophoresis (PAGE)

Native and urea PAGE were carried out using a low pH discontinuous gel system as outlined by Hames and Rickwood (1990) with slight modifications. Acrylamide gels (8.5 cm x 7.5 cm x 1.5 mm) were used with a 3% stacking gel and 15% separating gel. Protein samples (60 µg) were made to 50 µl with 140 mM acetic acid and 25 mM KOH, pH 4.3, with 0.25% glycerol and 0.025% methyl green. In order to maximize electrophoretic separation of the basic recombinant protein the polarity of the gels was reversed. Gels were then run at 35 mA per gel at 4°C for 2 h or just until the dye front ran off the gel. Urea gels were prepared as above with slight modifications. The stacking and resolving gels were made up to 8 M urea. The samples were also made up to 8 M urea prior to loading.

Prior to electroblotting native and urea gels were rinsed 3 times for 10 minutes each in modified Dunn's buffer (10 mM $NaHCO_3$, 3 mM Na_2CO_3, 0.1 % SDS). Gels were then electroblotted for 2h at 300 mA onto nitrocellulose and probed with anti-BN28 antibodies as previously outlined for SDS-PAGE.

P) Relative Size Determination

Comparison of rBN28 and BN28 from 4 week acclimated leaf protein sample was carried out through size exclusion chromatography. Acclimated leaf sample (30 mg/ml) extracted in 50 mM Tris-HCl, pH. 8.0 and 1.0 mM PMSF and rBN28 (15 µg/ml) in the same buffer were separated on a G-50 Sephadex (Pharmacia,

Uppsala, Sweden) column 40 cm long with 1.5 cm diameter equilibrated with 25 mM Tris-HCl, pH. 8.0. Samples (0.5 ml) were loaded on the column. Following a 25 ml void volume, 1.5 ml fractions (36) were collected. Samples of each fraction (25 µl) were mixed with 30 µl SDS-loading buffer, run on a Tris-Tricine gel, electroblotted onto nitrocellulose and probed with anti-BN28 antibody as previously described.

Q) Isoelectric Focusing

Isoelectric focusing (IEF) was carried out as described in the procedures provided with Isogel Agarose IEF Plates (FMC, Rockland, U.S.A). Acclimated leaf protein (20 mg/ml) extracted in 25 mM Tris-HCl, pH.8.0, and purified rBN28 (0.88 mg/ml) was analyzed. Samples (5 µl) were loaded on a 7 cm x 10 cm isogel agarose IEF plate (pI 3-10) and focussed at 5 W for 5 min and then at 20 W for 90 min. Proteins were press bolt transferred overnight to 0.22 µm nitrocellulose (Schleicher and Schuell, Keene, U.S.A), equilibrated in TBS (10mM Tris-HCl, pH 7.4 and 150 mM NaCl) and then probed with anti-BN28 as outlined for immunoblotting.

R) 2-D IEF/SDS-PAGE

Two-dimensional PAGE were carried out as described by Monroy et al. (1993) with slight modifications. IEF of proteins was undertaken using a gel mixture containing 4% acrylamide, 8 M urea, 5% ampholytes (2% pH 4-6; 2% pH 5-7; 1% pH 3-10) and 0.4% Nonidet-40. Soluble proteins from 42 day acclimated leaf tissue were extracted as outlined for native and urea gels. Soluble protein

samples (approximately 20 µg/µl) were diluted to 8 µg/µl with 2D-IEF loading buffer (9.5 M urea, 5% ampholytes (2% pH 4-6; 2% pH 5-7; 1% pH 3-10), 5% β–mercaptoethanol, and 2% Nonidet-40). Following pre-focusing of the tubes (200 V for 15 min; 300 V for 30 min; 400 V for 30 min), 400 µg acclimated proteins were focussed for 18 h at 400 V. Tubes were removed and equilibrated 2 times for 15 min each in SDS-loading buffer. Following equilibration, tubes (9.0 cm x 1.5 mm) were placed on a Tris-Tricine gel lacking a stacking gel (8.5 cm x 6.5 cm x 1.5 mm) and run as previously outlined. After running the second dimension, the proteins were electroblotted onto nitrocellulose and probed with anti-BN28 polyclonal antibodies.

S) Staining for Glycoproteins

Protein from acclimated leaf tissue was extracted in 50 mM Tris-HCl, pH. 8.0 and 1.0 mM PMSF. Protein concentration was determined as outlined in section C) and the protein sample was diluted to 20 mg/ml with the same Tris-HCl buffer. The sample (1 ml) was then boiled for 10 min, placed on ice for 15 min and centrifuged at 13500 x g for 20 min to remove the boiling insoluble polypeptides. To further concentrate the sample the supernatant was removed and the proteins precipitated with 8 volumes of 100% acetone at -20°C. Following 1.5 h incubation at -20°C, the peptides were centrifuged at 5000 x g for 15 min. The pellet was then resuspended in 80 µl of SDS-PAGE sample buffer and boiled. Samples (30 µl/lane containing 3.75 µg BN28) were separated on a 16% Tris-Tricine gel as previously outlined in section D. Staining for glycoproteins was performed as outlined by Hames and Rickwood

(1990). Following separation, the gel was submerged in 25% 2-propanol and 10% acetic acid for 2 h. The solution was replaced and the gel was stored overnight in fresh solution. The following morning the solution was removed and the gel was soaked in 25% 2-propanol, 10% acetic acid and 0.2% thymol for 90 min. This was then replaced with 80% sulfuric acid and 20% ethanol for 2 to 3 h. Over this period the gel became opaque and then clarified revealing glycoproteins as red/pink bands.

T) Evaluation of Freezing Tolerance

Freezing tolerance was evaluated using an electrolyte leakage assay (Sukumaran and Weiser, 1972; Boothe et al., 1995; Singh, 1995). Leaves were harvested and washed with distilled water. Disks of 1 cm diameter were punched from leaves, avoiding the midrib, with a cork borer and placed on moistened filter paper in small petri dishes (2-3 disks/dish). The petri dishes were placed in a programmable freezer and the temperature lowered to 0°C and held for 1 h, at which time the first set of samples was removed and transferred to 4°C. The temperature was then lowered to -2.5°C and ice crystal nucleation initiated by touching a small chip of dry-ice to the edge of the moistened filter paper. Samples were maintained at -2.5°C overnight allowing them to reach thermal equilibrium. The temperature was then lowered at a constant rate of 2.5° h^{-1} and samples collected at 2.5°C intervals between -2.5°C and -17.5°C. All samples were allowed to thaw at 4°C for at least 12 h prior to determining electrolyte leakage.

Electrolyte leakage brought about by freezing was determined

by placing samples along with the accompanying filter paper in tubes containing 10 ml of glass-distilled water, shaking overnight at room temperature and measuring conductivity with a CDM 83 conductivity meter (Bach-Simpson, London, Canada). Total electrolytes in the leaf tissue were determined after boiling for 3 min, to disrupt all cells, cooling to room temperature for 1 h with shaking, adjusting the volume to 10 ml and re-measuring the conductivity. Electrolyte leakage was expressed as a percentage of the total (Sukumaran and Weiser, 1972). Freezing tolerances are expressed as the temperature causing 50 % ion leakage (TL_{50}).

U) Chlorophyll Determination

Chlorophyll a/b ratios were determined as outlined by Vernon (1960), with slight modifications. Leaf tissue (100 mg) was ground with mortar and pestle in liquid nitrogen. Tissue was resuspended in 500 µl 80% aqueous acetone, mixed thoroughly and centrifuged at 13500 x g for 10 min. The supernatant was removed and kept while the pellet was resuspended in a second 500 µl volume of acetone. After centrifuging at 13500 x g, the second supernatant was removed and combined with the first. The combined acetone fraction containing the chlorophyll was diluted to 5 ml with 80% acetone and read on the spectrophotometer at 649 nm and 665 nm. From these values, chlorophyll a, b, and a/b were calculated.

V) RNA Extraction and Northern Blot Analysis

RNA extraction and Northern blotting were carried out as outlined by Sambrook et al. (1989) with slight modifications. Leaf

tissue (1 g) was ground to a fine powder with a mortar and pestle while frozen under liquid nitrogen. All operations were performed on ice and centrifugations were carried out at 4°C, unless otherwise indicated. RNA extraction buffer (5 ml), consisting of 4 M guanidinium thiocyanate, 2% (w/v) sarcosyl, 50 mM Tris-HCl, pH 8.0, 10 mM EDTA and 1% 2-mercaptoethanol, was added. Grinding continued until a thawed slurry formed. The slurry was transferred to a centrifuge tube and 0.2 volumes of 10% (w/v) SDS, 0.1 volume of 2 M sodium acetate (pH 4.0), 1.0 volume of phenol and 0.2 volumes of chloroform: isoamyl alcohol (24:1) were added. The mixture was vortexed vigorously and incubated at room temperature for 15 min with agitation before centrifuging at 7500 x g for 20 min. Isopropanol (0.8 volumes) was added to the aqueous phase and RNA precipitated on ice for at least 1 h followed by centrifugation at 7500 x g for 20 min. The pellet was redissolved in DEP-treated water and the RNA was precipitated with an equal volume of 6 M lithium chloride at 4°C for at least 12 h. After centrifugation at 7500 x g for 20 min, the RNA pellet was rinsed with 2 M lithium chloride followed by 70% ethanol and then dissolved in DEP-treated water. The RNA concentration was determined spectrophotometrically from the absorbance measured at 260 nm. Total RNA (20-40 µg) was separated on agarose-formaldehyde gels according to standard protocols (Sambrook et al., 1989). RNA was subsequently capillary-transferred to Zetaprobe (BioRad, Oshawa, Canada) membranes and hybridized with probes constructed from the full length (195 bp) *Brassica napus* cDNA clone representing the BN28 gene. The probes were radiolabelled with [α-^{32}P]dCTP (Amersham, Oakville, Ont.)

using a multiprime labeling system, following the manufacturer's instructions (Amersham, Calgary, Canada). Hybridizations were carried out in 50% formamide, 0.12 M NaH_2PO_4, pH 7.2, 0.25 M NaCl, 7% SDS, 1mM EDTA at 42°C, and the final wash was in 0.1 x SSC, 0.1% SDS at 65°C. *bn*28 mRNA levels were compared in leaves 1 and 4 of both 0 day and 8 day acclimated seedlings.

W) Immunocytochemistry

Tissue sections ($1cm^2$) were fixed overnight under vacuum in FAA (4% formaldehyde final concentration from 37% stock solution, 50% ethanol, 5% acetic acid), followed by dehydration through a graded tert-butanol (TBA): ethanol series (10%:40%, 20%:50%, 35%:50%, 55% :45%, 75%:25%) for 1.5 h per step. The tissue was rinsed 3 times for 30 min each with 100% TBA and then left overnight in 1:1 TBA:paraffin oil. The tissue was infiltrated in molten Paraplast Plus (Sigma, St. Louis, U.S.A.) at 65°C with two changes of fresh paraffin over a 24 h period, then embedded. The material was sectioned at 8 µm and placed on slides that were pre-treated with gelatin (0.5% gelatin, 0.05% $Cr(SO_4)_2.12H_2O$) then flooded with a 4% aqueous formaldehyde solution. Sections were adhered to the slides by incubating the slides at 35°C for 30 min., then removing the formaldehyde solution and drying overnight. Sections were deparaffinized with two washes of 100% xylene for 15 min, then were hydrated in a descending ethanol series (100%, 95%, 85%, 70%, 50%, 30%, 15%) for 15 min per step, rinsed in water then washed twice in TBS-T. Sections were etched with 0.3% H_2O_2 in TBS-T and then blocked for 15 min in TBS-T with 5% BSA (w/v).

Following rinsing in TBS-T, the slides were left overnight in primary antibody diluted 1:200 with TBS-T and 0.3% (w/v) BSA. After rinsing 3 times for 10 min in TBS-T to remove excess primary antibody, the slides were incubated for 2 h in 1:200 diluted goat anti-rabbit IgG (1:200) conjugated with alkaline phosphatase (Sigma, St. Louis, U.S.A.). To remove excess secondary antibody the slides were washed twice for 5 min with TBS-T followed by 2 washes with TBS. The colour reaction was carried out as described for immunoblotting.

X) Immunolocalization

Segments ($<1mm^2$) from the fourth leaf of four-leaf-stage seedlings were fixed for 1.5 h in 2% glutaraldehyde in phosphate buffer (50 mM Na_2HPO_4, pH 7.4) at room temperature. After rinsing the tissue with phosphate buffer twice for 15 min, the segments were fixed at room temperature in 1% OsO_4 in phosphate buffer. The tissue was washed twice for 15 min in phosphate buffer and then dehydrated in an ascending series of ethanol (0%, 20%, 40%, 60%, 80%, 100%) for 30 min per step. Tissue infiltration was done with a 30 min rinse in 1:1 ethanol: LR white resin followed with 100% LR white overnight. The LR white (London Resin, Basingstoke, U.S.A.) was changed twice the following day, then the segments were put into gelatin capsules with LR white and polymerized overnight at 60°C. Gold/silver sections were cut using a Reichart ultramicrotome (AO Instrument Company, Buffalo, U.S.A.) and glass knives. Sections were collected on 300 mesh nickel grids coated with 2.5% (w/v) Formvar in acetone (Marivac, Halifax, Canada). The sections were

etched by floating the grids on 10%(w/v) sodium metaperiodate in TBS for 15 min. Grids were washed twice for 10 min in TBS and once for 10 min in TBS-T, then floated in TBS-T with 0.5% BSA and 0.2% fish skin gelatin for 10 min to block nonspecific binding. Thereafter, grids were incubated with rabbit anti-BN28 primary antibody for 1 hour at room temperature, washed twice for 10 minutes on TBS-T then incubated on 1:10 goat anti-rabbit IgG conjugated to 10 nm gold particles (Sigma, St. Louis, U.S.A.) for 1 h at room temperature. After rinsing the grids twice with TBS-T for 10 min, and once on water, the grids were stained for 10 min in 4% uranyl acetate and given a final rinse with water. Sections were viewed on a EM 201 (Phillips, Eindhoven, Nederlands) at 80kV.

Y) Differential Centrifugation

Differential centrifugation technique were carried out as outlined in Anderson et al. (1995). Leaves from low temperature-treated plants were ground with a mortar and pestle on ice, in either low-salt (50 mM Tris-HCl pH 8.0, 50 mM NaCl, 1 mM PMSF) or high-salt (50 mM Tris-HCl pH 8.0, 500 mM NaCl, 1 mM PMSF) buffer The extracts were centrifuged at 9,000 x g for 20 min and the organellar membrane pellets saved. An aliquot was taken from each supernatant and the remainder centrifuged at 100,000 x g for 1h to isolate microsomal membranes. The pellets from this and from the previous centrifugation were resuspended in 50 mM Tris-HCl, pH 8.0, 2% SDS and 1 mM PMSF and vortexed vigorously. Similarly, both low- and high-speed supernatants were made to 2% SDS and 1 mM PMSF. All samples were quantified for protein, mixed with SDS-

PAGE sample buffer, heated in a boiling water bath for approximately 5 min and loaded onto a SDS-PAGE gel.

Z) BN28 Stability During Freeze and Post-Stress Recovery

BN28 stability was followed in leaf 4 of 4 week acclimated seedlings. Whole plants were placed at +2.5°C for 1h to reach equilibrium at which time the temperature was decreased at 5.0°/h to -7.5°C. Plants were held at -7.5°C for 3, 6, or 9 hours followed by a 4, 8, or 12h recovery period at 4°C. Proteins were extracted from leaf 4 and separated by SDS-PAGE, transferred to nitrocellulose and used for Western blotting.

a) Environmental Stresses and ABA Treatment

All environmental stresses were applied to seedlings at the four leaf stage. Drought stress was carried out as per Guo et al. (1992) and salinity stress according to Kurkela and Borg-Franck, 1992). Salt stress was applied by watering daily with a 450 mM solution of NaCl for 14 days. Drought treatment was induced by withholding water from seedlings. When wilting was observed the samples were collected and the relative water content was calculated as described by Guo et al. (1992). ABA treatments were applied as outlined by Orr et al. (1992). ABA-treated plants were watered daily with a 100 µM solution of ABA (mixed isomers, Sigma) for 14 days.

Results

A) Protein and Antibody Production

i) rBN28 Production and Purification.

The ligation of the BN28 gene into the polylinker site of pMAL-c (Fig. 2) disrupted the malE-lacZα fusion eliminating the expression of lacZα. If this construct is transformed into any lacZα-complementing strain of bacteria it will produce a white phenotype when grown on LB-amp with IPTG and X-gal. Conversely, pMAL-c vectors without an insert will continue to express lacZα and produce a blue bacterial phenotype when grown on X-gal and IPTG. This difference was used as a selection criterion following transformation of competent D5Hα *E. coli*. Following transformation and overnight growth in the presence of IPTG and X-gal, approximately 30-40% of the transformed colonies produced a white phenotype. Phenotypes of 20 to 30 colonies were re-tested by replating and again incubating overnight. Those transformants showing a white phenotype were used in the production of fusion protein.

The fusion protein was initially produced when liquid LB-AMP was inoculated with an overnight culture of transformed bacteria and incubated for three hours at 30°C with a minimal amount of IPTG (0.1 mM). Following lysis, both the soluble and insoluble fractions were tested on SDS-PAGE for the presence of the fusion protein. It was observed that the MBP/rBN28 fusion protein accumulated in the soluble fraction.

High expression of polypeptides in bacteria often can lead to the formation of inclusion bodies appearing in the insoluble protein fraction and requiring strong denaturants such as guanidine hydrochloride to isolate the protein of interest. Since the fusion protein was found in the soluble fraction, the isolation of the peptide was simplified.

In an attempt to optimize the production of fusion protein, varying IPTG concentrations were tested. Bacterial cells were induced with 0.1 mM, 0.2 mM and 0.3 mM IPTG. It was observed that the various IPTG concentrations tested had no effect on the accumulation of MBP/rBN28 (Fig. 3).

The effects of varying growth temperature was also addressed. Growth at temperatures lower than 37°C reduced the amount of soluble fusion protein while growth at higher temperatures was detrimental to bacterial growth (data not shown).

Finally, to optimize the production of fusion protein, the bacterial culture was induced for 1 to 3 hours with 0.3 mM IPTG at 37°C. Very little fusion protein was produced after 1 hour of induction (Fig. 3). However, by 2 hours of induction a significant increase in MBP/rBN28 fusion protein was observed. By 3 hours of induction, the fusion protein accounted for approximately 30% of the total soluble bacterial protein. Growth for longer periods of time did not increase the amount of soluble fusion protein. Therefore, the conditions that were utilized are growth at 37°C and induction with 0.1 mM IPTG for 3 hours.

Transformed *E. coli* induced with IPTG synthesized an approximately 45 kDa polypeptide corresponding to the maltose

binding-BN28 fusion protein (Fig. 4) (upper arrow). After proteolytic cleavage of induced bacterial proteins there was an observable decrease in size of the fusion protein (lower arrow) corresponding to the separation of rBN28 (6.5 kDa) from the maltose binding protein (39 kDa). rBN28 was not detected by Coomassie stain because of the amino acid composition of the small peptide. A silver stained gel of induced bacterial proteins revealed the presence of rBN28 and other low molecular weight proteolytic fragments following cleavage (Fig. 5, lane Ic).

Purification of the MBP/rBN28 fusion protein utilizing amylose affinity chromatography was unsuccessful. A large amount of protein extracted in both high (250 mM NaCl) and low (25 mM NaCl) salt buffers passed directly through the column when applied to the amylose resin (Fig. 6). Following rinses to remove residual peptides, maltose was added to competitively elute the bound peptides. Very few peptides were detected in the eluted fractions. To ensure that the fusion protein did not remain on the column, a small sample of amylose resin was run on SDS-PAGE to determine the presence of bound fusion protein. No fusion protein was detected with the amylose resin suggesting that the fusion protein was not binding to the resin. Therefore, the fusion protein could not be separated from the other bacterial proteins. The bacterial proteins extracted in higher salt (250 mM NaCl) showed very limited binding of the fusion protein to the column. However, not nearly enough fusion protein was bound to the column to be used in further experiments or in the production of antibodies. Therefore, an alternate approach was undertaken.

The alternate approach utilized differences in pI between rBN28 and the fusion protein. The basic pI of rBN28 (9) allowed for the use of anion exchange chromatography to purify rBN28 from the other bacterial polypeptides. DEAE cellulose at pH 8.0 bound negatively charged proteins including the MBP/rBN28 (pI<8.0) fusion protein. Positively charged polypeptides were washed from the column. The factor Xa protease was added to the column to cleave the fusion protein. Following cleavage, positively charged rBN28 and other positively charged proteolytic fragments were removed from the column while the MBP, protease and other negatively charged fragments were retained. This purification isolated a product that appeared as a single heavy band, with a second weakly staining band of slightly lower molecular mass, on an SDS-PAGE gel following silver staining (Fig. 5, lane P). Initial concerns that the lower mass polypeptide was bacterial in origin were dismissed with the results of the molecular weight determination and amino acid composition of the purified product which matched the predicted values for BN28. Therefore, the apparent doublet was probably an artifact of the gel system. BN28 isolation began with 500 ml of induced bacterial cells. Following binding to DEAE cellulose and protease treatment, 6.1 ml was removed from the column containing 5.3 mg of rBN28 as quantified spectrophotometrically by absorbance at 205 nm utilizing Beer's law (Table 2).

ii) Antibody Production and Purification

BN28 was expected to have low antigenicity on the basis of its small size and hydrophilic nature. Titering of the antibodies

revealed that 1:50 dilution was required to detect BN28 on a Western blot. Following 2 more injections the titre was increased to 1:300.

To minimize non-specific binding on Western blots and in immunolocalization studies, the polyclonal antibodies were purified. Fig. 7 shows a Western blot of acclimated leaf proteins probed with pre-immune, purified pre-immune, immune and purified immune serum. For both pre-immune and immune sera there is a noticeable amount of nonspecific binding of antibodies to other proteins. However, after purification, the nonspecific binding was eliminated without reducing the affinity of the purified immune serum antibody to BN28.

B) Biochemical and Biophysical Analysis of BN28 and rBN28

i) Secondary Structure Predictions

Predictions of rBN28 secondary structure were based on the derived amino acid sequence for BN28 and standard algorithms (Chou and Fasman, 1978 and Robson and Garnier, 1978). Prediction of secondary structure was derived from a moving 7-residue window. Results from the Chou and Fasman (1978) algorithm suggest that rBN28 consists of α–helix from amino acid 15 through 52, a small amount of β-sheet from residue 53 to 59 and turns at both the C- and N- terminal residues (Fig.8). Conversely, the algorithm by Robson and Garnier (1978) suggest that rBN28 is α–helical from residue 9 through 52, with no apparent β-sheet or turns in the protein and random coil configuration at the two ends

(residues 1-9 and 53-65). A consensus region of α-helical structure was predicted for the central portion of rBN28 from residues 15 to 52 (Fig. 8) with random coil at the N and C ends of the peptide. Comparison of the predicted secondary structure for rBN28 with the deduced secondary structure for antifreeze proteins showed similarity with type I antifreeze proteins, which are exclusively α-helical.

ii) Secondary Structure of rBN28

To compare the structure of rBN28 with the known secondary structure of antifreeze proteins, the secondary structure of rBN28 was studied utilizing CD and NMR (Boothe et al., 1997). CD was utilized to measure the ellipticity of rBN28, as measured in degrees, when scanned from 190 nm to 250 nm. The observed ellipticity was then converted into molar ellipticity by multiplying the observed value by the mean residual molar weight (molecular weight divided by the number of residues) and dividing by 10 times the peptide concentration in g per ml. Fig. 9 shows the molar ellipticity for rBN28 scanned from 190 nm to 250 nm both in the presence and absence of trifluoroethanol (TFE). Deconvolution of the CD spectra using the RBOcon algorithm (Sonnichsen et al., 1992) indicated that rBN28 was composed of 13.5% α-helical, 0% β-sheet, 22.5% turns and 64% random coil (Fig. 9, A). Since a large amount of rBN28 was expected to be α-helical, rBN28 was re-examined in the presence of varying concentrations of TFE, which is known to induce α-helical conformation in peptides (Sonnichsen et al., 1992). The addition of 50% TFE increased the amount of helix from 13.5 % to 74.5 % along

with 0% β–sheet, 0% turns and 25.5% random coil at 50% TFE concentration (Fig. 9, B). However, when the TFE was removed from the rBN28 the peptide reverted back to its original conformation suggesting that the induced α– helix is unstable (Fig. 9, C). Similar results were observed at both 5°C and 25°C. Boothe et al. (in press) corroborated the CD results through one- and two-dimensional NMR-spectroscopy of rBN28 at 5°C and 25°C. They revealed the presence of signal degeneracy in the 1D-NMR-spectrum. This together with a lack of sequence dependent frequency dispersion between amino acids and the statistical analysis of αCH and HN-proton frequencies. suggested that rBN28 lacked any regular secondary structure and was 100% random coil. The lack of secondary structure in rBN28 prevents it from being a type I antifreeze protein since helical conformation is required for proper hydrogen bonding of the peptide to the surface of the ice crystal.

iii) Determination of BN28 Concentration in Acclimated Leaf Tissue During a Freeze Stress

During a freeze stress there is a loss of cytoplasmic water that leads directly to an increase in the cellular concentration of solutes, including BN28. Therefore, unfrozen water content was determined over the temperature range of +2.5°C to -17.5°C (Fig. 10). The integrated area under the NMR peak for unfrozen water was determined for acclimated leaf tissue held at +2.5°C. As the tissue was frozen the amount of unfrozen water in the leaf decreased as indicated by a decrease in the area under the NMR curve for unfrozen water. Following measurements at -17.5°C, the total water

in the leaf tissue was determined. The ratio of the integrated areas at the various temperatures and at +2.5°C ($A_T/A_{2.5}$) were combined with both the fresh and dry weights for each leaf to calculate the total grams of unfrozen water per gram dry weight leaf tissue over the temperature range. Results showed that the freezing of water began upon ice nucleation at -2.5°C. At -2.5°C the acclimated leaf contained 5.2 grams water per gram dry weight. As the temperature decreased there was a rapid loss of unfrozen water. By -5.0°C only 1.5 grams water per gram dry weight remained. From -5.0°C to -17.5°C the unfrozen water content decreased slightly to 0.5 grams. Therefore, at -17.5°C less than 10% of the water in the tissue was in the unfrozen state.

To determine the physiological range of BN28 concentrations, the amount of BN28 in acclimated leaf tissues needed to be measured. Boothe et al. (1995) have shown that after 8 days acclimation, leaf 4 of four leaf stage seedlings contains 0.414 µg BN28 per mg protein, and by 42 days acclimation this value has increased to 0.494 µg BN28 per mg protein. To calculate the cellular concentration of BN28 the amount of BN28 (0.494 µg BN28 per mg protein) was multiplied by the total protein measured in the leaf tissue (419.36 mg total protein per gram dry leaf weight) and then divided by the water content at the various temperatures (grams of water per gram dry leaf weight). This provided values for BN28 concentration in µg BN28 per gram water. This was converted into molar values using 6500 Da as the molecular weight of BN28. The calculated values for BN28 concentration was plotted over the temperature range of the study (Fig. 11). From this figure in was

shown that the physiological concentration of BN28 increased one order of magnitude from 6.2 µM at +2.5°C to 67 µM at -17.5°C. This provided a physiological range of concentrations that were used in other studies.

iv) Recrystallization Inhibition Activity of rBN28

To determine if BN28 was an AFP, its ability to inhibit or alter the formation of ice crystals in a "splat cooling assay" was addressed (Hightower et al., 1991). The addition of physiological amounts of rBN28 to solution had no effect on ice crystal formation. When rBN28 was increased to well above the physiological levels for BN28, again there still was no effect of ice crystal formation. Therefore, rBN28 probably does not have recrystallization inhibition activity.

v) Cryoprotection

Boothe et al. (in press) have shown that BN28 remains in solution when boiled. This is characteristic of several low temperature-inducible peptides. To obtain a better understanding of the boiling and freezing stability of BN28 the predicted hydrophilicity was determined for the peptide utilizing the standard algorithm of Kyte and Doolittle (1987). Using a moving 7-residue window it was predicted that BN28 is highly hydrophilic containing small amounts of hydrophobic regions interspersed throughout the peptide.

The stability during a freeze stress and the hydrophilic nature of the protein together with the random coil configuration of BN28 are all characteristics of a cryoprotective compound. To determine if

rBN28 has cryoprotective properties rBN28 and other solutes were tested in an *in vitro* assay in which the ability to prevent freeze denaturation of the enzyme lactate dehydrogenase (LDH) was followed spectrophotometrically (Fig. 12, a). Plotting residual LDH activity, following an overnight freeze at -17°C, against the concentration of the compound studied revealed that BN28 provided 50 % cryoprotection (CP_{50}) at 3.2 µg/ml, much better than BSA (CP_{50} of 12.5 µg/ml) and provided greater cryoprotection than both sucrose (CP_{50} of 16,000 µg/ml) and proline (CP_{50} of 100,000 µg/ml), 2 known cryoprotectants (Table. 3). However, on a nM basis, BSA is slightly better (CP_{50} of 200 nM) than rBN28 (CP_{50} of 500 nM) but rBN28 is still a better cryoprotectant in relation to sucrose and proline (Table 3). Fig. 12 also shows that over the physiological range of BN28 concentrations, represented by the two vertical lines, rBN28 can maintain 80% to 100% LDH activity in this *in vitro* assay. Following an overnight freeze stress, an aqueous solution with LDH containing no cryoprotectant retained 3% of the initial LDH activity. The addition of rBN28 to a solution containing LDH, following a freeze thaw stress, could not return LDH activity to the original levels used. Also, a solution containing LDH held overnight at 4°C retained only 67% of the initial LDH activity.

rBN28 cryoprotective activity was also studied using G6PDH, from non-acclimated leaf tissue, in an *in vitro* assay. Initial G6PDH activities were measured and designated a value of 100%. A solution containing G6PDH held overnight at 4°C retained only 72% of the initial G6PDH activity. Following an overnight freeze, solutions containing no cryoprotectant retained only 16% G6PDH activity

(Fig. 12, b). It was shown that over the physiological concentration range for BN28 (40 µg/ml to 400 µg/ml), rBN28 maintained 66% to 82% of the initial G6PDH activity.

vi) Further Structural Considerations of BN28

To address the question of possible post-translational modifications of BN28 both native and urea PAGE are undertaken. Native PAGE separates proteins based on their charge to mass ratio. Urea PAGE also separates peptides on their charge to mass ratios. However, the urea eliminates the quaternary structure, thereby providing information on the presence of multimeric compounds or protein/protein associations.

rBN28 was resolved as a single band on a non-denaturing native gel. However, BN28 from 4 week acclimated leaf tissue was resolved into 3 distinct bands all migrating more slowly than the recombinant peptide (Fig. 13,A). Boiling had no effect on the banding pattern.

When the same samples were separated on a urea gel, rBN28 appeared as a single band while BN28 from acclimated leaf tissue appeared as 3 distinct bands. Similar results obtained on urea and native gels suggested that BN28 is not part of a multimeric structure (Fig. 13,B). However, the differences in mobility between rBN28 and the 3 peptides detected from the acclimated leaf sample suggest that rBN28 undergoes alterations either increasing the overall size or making BN28 more negatively charged.

Size exclusion chromatography was used to examine possible molecular weight differences between both rBN28 and BN28

acclimated leaf proteins. A column packed with Sephadex, G50 was standardized with proteins of known molecular weight and a calibration curve was produced for the column (Fig. 14). The calibration curve shows that the column can effectively separate peptides in the range from 6.0 kDa to 25 kDa. This is well within the range required to study differences in molecular weight between BN28 and rBN28. The sample of rBN28 and the acclimated leaf proteins were loaded on the column. Following a 25 ml void volume, fractions were collected. The collected fractions were separated on an SDS-PAGE gel after which the gel was utilized for Western blotting. Analysis of the Western blots revealed that both rBN28 and the native BN28 eluted from the column in similar fractions (Fig. 15). This suggested that no detectable size difference existed between rBN28 and BN28.

The molecular mass of rBN28 was determined utilizing mass spectroscopy (Boothe et al., in press). Samples (100-250 pmol) were applied to a nitrocellulose-coated mylar disk in a 50:50 mixture of 0.05% aqueous trifluoroacetic acid and ethanol. Following absorption and spin-drying, the spectra were collected for 3 hours at 16 kV. The value of 6422.8 Da was obtained as the molecular weight for rBN28. The value obtained from mass spectrometry was in close agreement with the value of 6420.5 Da predicted from the derived amino acid sequence (minus the N-terminal methionine).

Amino acid analysis of rBN28 following acid hydrolysis in 6N HCl with 0.1% phenol at 110°C for 24 hours revealed that the major constituents of rBN28 were alanine (22%), lysine (12%), glycine (12%), glutamine (7.7%), serine (11%) and threonine (11%). rBN28

was also shown to lack histidine, tyrosine, proline and cysteine (Fig. 16).

To address possible alterations in size and charge, rBN28 and acclimated leaf proteins were separated utilizing 1-dimensional isoelectric focusing gels (Fig. 17). Analysis of pI on a gel with a range of 3-10 revealed that the apparent pI for rBN28 is 9.1, which was close to the predicted pI of 9. However, the pI of BN28 in acclimated leaf tissue was 6.4, suggesting the protein underwent post-translational modifications that altered the net charge.

Since only one band was distinguishable for BN28, IEF/SDS-PAGE/Westerns were done on soluble proteins from 42 day acclimated leaf tissue (Fig. 18). This procedure resolved two distinct peptides, and possibly a third. The two clearly visible peptides differed in both size and charge. This technique is limited by the amount of sample that can be loaded on the capillary IEF tube. After the first dimension was run it was observed that not all of the sample was focused on the capillary. Attempts to load 500 µg resulted in more of the sample not being focused.

Attempts to determine possible modifications to BN28 focused on protein glycosylation and phosphorylation. Analysis of the amino acid sequence of BN28 revealed 7 putative phosphorylation and 11 putative glycosylation sites (Fig. 16).

The possible glycosylation of BN28 was examined by staining for glycoproteins following separation of proteins from acclimated leaf tissue on SDS-PAGE. The low abundance of BN28 in acclimated leaf samples, well below what is needed to be detected by glycoprotein stains (3 µg), prevented the direct use of the protein

samples on SDS-PAGE. Therefore, BN28 was enriched in the sample through boiling and acetone precipitation followed by resuspension in 1/10 the original volume. When the gels were stained for glycoproteins 1 very prominent band was detected at approximately 100 kDa along with some other less intensely stained smaller peptides. However, BN28 was not stained, suggesting that it is not a glycoprotein (Fig. 19).

C) Temporal and Spatial Distribution of BN28 in Acclimating Seedlings

In an attempt to observe various levels of regulation that are involved in the accumulation of BN28, the temporal and spatial distribution of the polypeptide was studied.

i) Seedling Hardening

To determine if BN28 is involved in the acquisition of freezing tolerance or in metabolic adjustment to growth at low temperature, it was imperative to study the rate of acclimation and deacclimation through electrolyte leakage of leaf tissue. Ion leakage was monitored in leaf tissue frozen from -2.5°C to -17.5°C (Fig. 20). Non-acclimated leaf tissue had an TL_{50} of approximately -2°C. The greatest increase in TL_{50} occurred in the first 14 days of acclimation with TL_{50} value reaching -11°C. There was very little change in freezing tolerance between 14 days and 28 days acclimation. However, the freezing tolerance of the leaf tissue reached -16°C by 42 days low temperature exposure. Leaves from plants acclimated for 42 days and then deacclimated for up to a week at room temperature

maintained high levels of freezing tolerance. Leaf tissue that was deacclimated for 2 and 7 days still showed significant levels of freezing tolerance (TL_{50} values of -13°C and -10°C, respectively).

To characterize the steady state levels of BN28 during acclimation and deacclimation, Western blotting was undertaken in leaf 4 tissue of four leaf stage seedlings. Western blotting revealed that BN28 was detectable by 8 days of acclimation (Fig. 21). Attempts to detect BN28 in 4 day acclimated leaf tissue provided inconsistent results; some samples showed a faint band while others showed nothing. BN28 continued to increase over the next 20 days. Between 28 to 42 days acclimation only a slight increase in accumulation was observed. When the acclimated plants were returned to non-acclimating conditions, BN28 became undetectable between 2 and 7 days. Samples from 1 and 2 days deacclimated leaves showed the presence of a faint band below BN28. This was possibly a degradation product of BN28 that was detected on the Western blot. By 7 days deacclimation, this band was no longer detectable.

ii) Organ Specific Accumulation of BN28 in 21 Day Acclimated Seedlings

Polyclonal antibodies specific for BN28 were used to examine extracts of control and 21 day acclimated seedlings for possible BN28 accumulation in leaf blades, petioles, hypocotyls, roots, apical meristems and stem tissues. BN28 was not detected in non-acclimated tissue. The highest level of BN28 was found in the leaf blade and apical meristems (Fig. 22). There was little BN28 in the

petiole and hypocotyl and none was detected in root tissue from acclimated plants. Although high accumulation of BN28 was observed in the apical meristem, the stem tissue 10 mm proximal from the apical meristem showed very little BN28 (Fig. 23). Therefore, BN28 accumulates in growing tissues that are known to achieve a high level of freezing tolerance (Perras and Sarhan, 1989; Andrews and Morrison, 1992).

iii) Temporal Distribution of BN28 in Leaves of Acclimating Seedlings

Once it was shown that BN28 differentially accumulates in various plant organs the study focused on the effect of plant developmental stage and the accumulation of BN28 in leaves at different developmental stages. Differences in accumulation of BN28 were studied in seedlings at the first leaf stage, fourth leaf stage and seventh leaf stage (Fig. 24). After 21 days acclimation all the leaves on the plants were sampled separately (Fig. 25). One new leaf was initiated during the acclimation period. Hence the first leaf stage seedlings had 2 leaves, the fourth leaf stage had 5 leaves and the seventh leaf stage had 8leaves. Independent of seedling age, BN28 was always found to accumulate to the highest levels in the youngest developing leaf and decrease progressively with leaf age. BN28 was not found in the cotyledons. Since similar patterns of accumulation were seen in all plant ages, a single plant age (four leaf stage) was selected for further study.

iv) Electrolyte Leakage from Leaf 1 and Leaf 4 of Four Leaf Stage Seedlings

To determine differences in freezing tolerance, ion leakage was monitored in leaves 1 and 4 of four leaf stage seedlings that were acclimated at low temperature for 0 and 8 days. Under control conditions the TL_{50} of the two leaves was -2.5°C (Fig. 26). By 8 days of acclimation the freezing tolerance of leaf 1 was only -3.0°C. However, the freezing tolerance of leaf 4 had increased to -7.5°C. Therefore, the younger leaf appeared to have the greatest potential for freezing tolerance.

v) Northern Blot Analysis of *bn*28 mRNA from Leaves 1 and 4 of 21 Day Acclimated Seedlings

The expression of *bn*28 mRNA was examined in leaves 1 and 4 of seedlings acclimated over 8 days to address the role of post-transcriptional regulation on the accumulation of BN28 (Fig. 27). In both leaf 1 and leaf 4 of four leaf stage seedlings there was no expression of *bn28* until exposure to low temperature. Leaf 4 showed high levels of *bn28* mRNA by one day of acclimation, with little change thereafter. Although *bn28* transcript levels were low in leaf 1 following one day of acclimation, the levels reached that observed in leaf 4, by 8 days.

vi) Chlorophyll a/b Determination of Leaves 1 Through 4 of 21 Day Acclimated Seedlings

To determine if senescence was playing a role in the differential accumulation of BN28, chlorophyll a/b ratios were measured for all leaves on a 21 day acclimated seedling at the four leaf stage (Fig. 28). No significant difference in chlorophyll ratios was observed for the 4 leaves. These results suggest that the observed differences in BN28 accumulation are not associated with senescence and reflect absolute differences found in leaf tissue at different developmental stages.

vii) Immunological Controls

Purified pre-immune serum and purified immune serum were tested for specificity on non-acclimated and 21 day acclimated leaf tissue (Fig. 29). Very little colour was formed when non-acclimated tissue (Fig. 29, c, d) was probed with either purified pre-immune or immune sera, suggesting very little non-specific binding was occurring. Colour formation was observed in 21 day acclimated tissue probed with purified immune serum but was not observed with the purified pre-immune serum (Fig. 29, a). This confirms the specificity of the purified immune serum for BN28.

viii) Immunohistochemical Localization of BN28 in Acclimated Leaf Tissue

In an attempt to gain insight into the tissue specific distribution of BN28 during acclimation, immunohistochemical techniques were employed on leaf 4 and the apical meristem region of control and 21-day-acclimated four leaf stage seedlings (Fig. 30). BN28 was found in the spongy mesophyll, pallisade layer and phloem

tissue of acclimated leaves (Fig. 30, b). There was no detectable expression in the epidermis with the exception of the guard cells. The lack of BN28 accumulation in the epidermis suggests that BN28 is being regulated at the tissue level in leaves. BN28 was found throughout the tunica and corpus of the apical meristem (Fig. 30, d). It was also present throughout the ground tissue and epidermis of newly initiated leaves (Arrow). The presence of BN28 in the epidermis of newly initiated leaves and the lack of BN28 in the epidermis of leaf 4 suggests that once the leaf has reached a certain stage of development, BN28 no longer accumulates in the epidermis and is degraded. The cross-reacting protein was not detected in hypocotyl and petiole sections (data not shown), nor was it present in non-acclimated tissue (Fig. 30, a, c).

ix) Differential Centrifugation of Various Protein Extractions from Acclimated Leaf Tissue.

Analysis of the gene sequence suggested that BN28 has no transit peptide (Fig. 16). Differential centrifugation was utilized to determine possible associations with membranes. Proteins from acclimated leaves were extracted in high and low salt buffers and were centrifuged to obtain the 9000 x g and 100000 x g supernatants and pellets. The various samples were run on an SDS-PAGE and used for Western blotting (Fig. 31). The BN28 polypeptide always appeared in the supernatant fractions and was never detected in the microsomal membrane fraction (100,000 x g pellet) or the organellar membrane pellets (9000 x g pellet) suggesting that

BN28 is probably not associated with cellular-membrane fractions and is therefore found in the cytoplasm under non-stress conditions.

x) Immunolocalization

To determine the subcellular distribution of BN28 and to detect possible associations with cellular structures, transmission electron microscopy and immunolocalization techniques were utilized. Very little non-specific binding of gold particles was observed in non-acclimated control tissue (Fig. 32,a). BN28 was detected throughout the cytoplasm of acclimated leaf mesophyll cells (Fig. 32,b-d. Further analysis revealed no apparent association between BN28 and chloroplasts (Fig. 32,c,d), mitochondria (Fig. 32,d), and plasma membranes (Fig. 32,b), suggesting that BN28 is largely if not totally cytoplasmic.

xi) BN28 Stability

Peptide stability was assessed by observing BN28 in leaf tissue of plants frozen for up to 9 hours at -7.5°C followed by a post stress recovery period for up to 12 hours at 4°C. Plants subjected to a sublethal freeze for up to 9 hours showed severe loss of turgor. However, if left at 4°C following the freeze stress the plant fully recovered. Analysis of BN28 during the freeze stress and recovery period showed no apparent decrease in BN28 levels. This suggested that BN28 was quite stable during a freeze stress (Fig. 33).

xii) BN28 Accumulation in Response to Environmental Stresses and ABA Treatment

Drought and salinity treatments subject tissue to a dehydrative stress similar to that experienced during a freeze stress (Levitt, 1980). Polyclonal antibodies specific for BN28 were used to probe leaf 4 samples subjected to different stresses for the accumulation of the polypeptide. Leaf tissue subjected to 14 days of drought stress showed significant wilting. The relative water content of leaves stressed for 2 weeks was measured at 36%, well below the 98% value obtained from unstressed leaf tissue (Boothe et al., 1995). However, Western blots showed no accumulation of BN28 (Fig. 34). Similarly, leaf tissue from plants subjected to 14 days salt stress that were loosing turgidity and becoming chlorotic also showed no apparent BN28 accumulation.

Previous results have shown that the application of ABA to plant tissue can induce the accumulation of other low temperature inducible peptides (Hajela et al., 1990; Kurkela and Borg-Franck, 1992). ABA did not induce BN28 accumulation when the leaves were sprayed daily for 14 days or when a leaf cut at the petiole was dipped in a solution of ABA. Boothe et al. (1995) confirmed that ABA was reaching target sites within the cells and inducing a response by Northern blotting with a probe for an ABA specific gene (*rab16a*). Therefore, it appears that BN28 only accumulates in leaf tissue when exposed to low temperature (Fig. 34).

Discussion

Genes encoding low temperature inducible proteins have been isolated from a wide variety of plant species. These include the *cor* and *kin* genes in *Arabidopsis thaliana* (Gilmour et al., 1988; Lång et al., 1989), *cas* genes in alfalfa (Mohapatra et al., 1987a; Mohapatra et al., 1987b), *wcs* genes in wheat (Sarhan and Parras, 1987; Parras and Sarhan, 1989), *cor* genes in spinach (Guy and Haskell, 1987; Guy et al., 1988) and *bn* genes in *Brassica napus* (Orr et al., 1992; Weretilnyk et al., 1993). Little is known about the structure, function and *in situ* accumulation of the products of these. To consider the possible function of these proteins it is important to go beyond predictions of amino acid sequence, size and structure and begin to produce or purify the gene products, confirm the predictions and then begin to address functionality.

The purpose of this thesis was to gain insight into the physiological role of BN28 in *Brassica napus* cv. Cascade. This was accomplished by studying the accumulation of BN28 during low temperature acclimation conditions in combination with biophysical and biochemical studies of rBN28. The limited number of studies on low temperature-inducible proteins in literature makes it difficult to relate these results to the findings of others.

A) rBN28 Purification and Antibody Production

The physiological, biochemical and biophysical characterization of BN28 required the production and purification of BN28 and the

production of polyclonal antibodies. The initial question was, "What method should be used to purify BN28?"

The synthesis of low temperature inducible proteins has been previously accomplished utilizing *in vitro* transcription and *in vitro* translation (Lin et al., 1990; Weretilnyk et al., 1993; Chauvin et al., 1993). This combination of techniques has had limited success as it produces only µg quantities of the gene products, which are frequently not pure (Hajela et al., 1990). Therefore, this method was not considered for BN28 production. Cation- and anion-exchange and size exclusion chromatography were used to purify COR85 from cold-acclimated spinach leaf tissue (Kazuoka and Oeda, 1994). This approach was also not suitable because of the very low abundance of BN28.

If the cDNA has been isolated, recombinant DNA technology can be used to produce large quantities of protein utilizing a bacterial expression system. Ouellet et al. (1993) used this approach to over-express WSC200. The ability to produce large amounts of a peptide with such a system was critical in deciding to utilize this approach to produce sufficient BN28 for purification.

In the present study, the insertion of the full length *bn*28 cDNA into a bacterial expression system (Fig. 2) allowed for the production of mg quantities of a Maltose Binding/rBN28 fusion protein (MBP/rBN28) (Fig. 3). Unlike the purification of WCS200, which required boiling, SDS-PAGE and electroelution (Ouellet et al., 1993), the purification of BN28 did not require many purification steps.

It appeared that the isolation procedure for rBN28 produced a single peptide with a molecular mass slightly less than the expected

value as calculated from the predicted amino acid sequence. Similar discrepancies in molecular weight were observed for COR6.6 using a Tris-tricine SDS-PAGE system (Gilmour et al., 1996). Mass spectroscopy of rBN28 verified that the molecular weight of the recombinant form (6422.8 Da) corresponded to the predicted molecular weight for BN28 (6420.5 Da). Therefore, the inconsistency in the present study probably resulted from inherent inaccuracy in the gel system and not from errors in the purification procedure.

When the proteolytically cleaved products were separated on SDS-PAGE gels and stained, the rBN28 peptide could not be detected with Coomassie brilliant blue (Fig. 4). Increasing the amount of protein loaded per lane had no effect on the detection of rBN28. However, rBN28 was detected with silver staining (Fig. 5). It is understood that Coomassie brilliant blue interacts with both hydrophobic regions and positively charged regions of peptides (Harris and Bashford, 1987). The hydropathy plot for BN28 shows that the protein is highly hydrophilic (Boothe et al., in press) and lacks the hydrophobic amino acids tyrosine, tryptophan, cysteine and proline (Fig. 16). Therefore, protein hydrophilicity probably prevented the detection of BN28 with Coomassie blue.

Interestingly, COR6.6 has 69% amino acid homology with BN28 and yet COR6.6 is detected with Coomassie blue stain (Gilmour et al., 1996). Since COR6.6 contains only two extra hydrophobic amino acids, it seems unlikely that the addition of these would significantly alter the binding properties of the polypeptide.

a) Antibodies

To detect the steady state levels of BN28 in acclimating seedlings, polyclonal antibodies to rBN28 were produced. Antibodies to other low temperature-induced polypeptides have been reported in the literature (Lin and Thomashow, 1992b; Neven et al., 1993; Kazuoka and Oeda, 1994, Boothe et al., 1995). Polyclonal serum to COR15 of *Arabidopsis thaliana* was produced and utilized for Western blotting. Although the specificity of polyclonal serum to COR15 was shown, pre-immune serum was apparently not tested against plant proteins for detection of other proteins or non-specific binding (Lin and Thomashow, 1992b). Similar lack of controls were observed in other studies utilizing polyclonal antibodies (Neven et al., 1993; Kazuoka and Oeda, 1994).

In the present study, purification of the pre-immune and immune serums reduced the non-specific binding without affecting the affinity of the purified immune serum for BN28 (Fig. 7). This purification is imperative for immunocytochemical and immunolocalization techniques which are more prone to false positive results.

B) BN28 Function

On the basis of predicted sequence and structure, Kurkela and co-workers have suggested that KIN1 and KIN2 from *Arabidopsis thaliana* function as antifreeze proteins (Kurkela and Franck, 1990; Kurkela and Borg-Franck, 1992). All the members of the *kin* gene family are predicted to have high alanine content, to be hydrophilic

and to have a high degree of α-helical structure. As these are all features of type I AFP, my research explored the possibility that this family of proteins are AFPs.

The first question addressed was, "Does BN28 have an α-helical structure?" Both the Chou and Fasman (1978), and Robson and Garnier (1978) algorithms predicted that BN28 has an α-helical conformation (Fig. 8). However, results from circular dichroism (Fig. 9) and NMR studies (Boothe et al., in press) on rBN28 reveal that rBN28 is an unstructured protein consisting of 100% random coil when measured at 25°C. An increase in α-helical content of rBN28, following incubation in TFE, confirmed the propensity of rBN28 to take on α-helical structure (Fig. 9). However, when the solvent was removed, rBN28 returned to random coil configuration indicating that this secondary structure was unstable in aqueous solution (Fig. 9).

Structural analysis of antifreeze proteins of winter flounder and larvae of the beetle *Dendroides canadensis* expressed in transgenic tobacco suggested that AFP secondary structure is temperature dependent (Wu et al., 1991; Ananthanarayanan and Hew, 1977; Kenward et al., 1993). The CD spectra from winter flounder type I AFP showed a temperature-dependent change in the secondary structure of the peptide, decreasing from 85% α-helix at -1°C to almost completely random coil configuration at or above 70°C. This prompted the reexamination of rBN28 at 5°C. The results showed the peptide was still a random coil (Fig.9), thus confirming the lack of α-helical structure.

The apparent discrepancy between the predicted secondary structure for BN28 and the secondary structure for rBN28 obtained experimentally is most likely a result of inherent error in the algorithms. Chou and Fasman (1978) showed that this algorithm predicted regions of myogen to be α-helical. However, CD of these regions found them to be random coil. The algorithms used are only strong estimators of conformation based on statistical probabilities of secondary structure. Since rBN28 is quite small and contains a high number of one residue, it is possible to bias the algorithms and obtain an erroneous predicted secondary structure. Conversely, the CD and NMR both provide actual physical data of protein structure which are much more conclusive than predictions. Since both CD and NMR techniques suggest random coil configuration, this provides very strong evidence that rBN28 is probably random coil and not α-helical, as predicted.

The NMR and CD studies provided information regarding the recombinant form of rBN28, which may not reflect the secondary structure for the native BN28. However, the molecular mass and amino acid composition for rBN28 were exactly as predicted for BN28. This suggests that rBN28 is not a truncated or altered form of the native protein. Therefore, one would expect BN28 to also be random coil.

In the literature, suspected AFPs are tested for recrystallization inhibition (DeVries and Lin, 1977; Hightower et al., 1991; Griffith et al., 1992). To determine if BN28 functions as an AFP, recrystallization inhibition was examined and the peptide was found to have no effect on the formation of ice crystals.

Another characteristic of thermal hysteresis factors in plants (Hon et al., 1994) and AFPs in fish (DeVries and Lin, 1977) is their extracellular accumulation and function. The results from the immunolocalization study (Fig. 32), together with the apparent lack of a signal peptide, suggest that BN28 accumulates in the cytoplasm. Therefore, the cumulative results (the lack of α-helical structure, together with the inability to alter ice-crystal formation and the cytoplasmic localization of the peptide) all preclude BN28 from functioning as a type 1 AFP.

Another possible function for BN28 is as a cryoprotectant. Two theories have been proposed to explain how cryoprotectant molecules might function. One theory of cryoprotection deals with compounds that enhance the stabilization of membranes (Volger and Heber, 1975; Santarius and Giersch, 1983) while the second theory of cryoprotection involves compounds that lead to protein stabilization (Carpenter and Crowe, 1988). As results from differential centrifugation (Fig. 31) and sub-cellular localization (Fig. 32) studies showed that BN28 was not associated with plasma- or organellar membranes, it is unlikely that it protects membranes. This is supported by recent work addressing the cryoprotective properties of COR6.6 on isolated liposomes membranes (Webb et al., 1996; Uemura et al., 1996). Isolated liposomes containing COR6.6 were subjected to a dehydrative stress induced by freezing or osmotic stress. During the stress, the liposomes were measured for an increase in incidence of either freeze-induced fusion and leakage of liposomes (Uemura et al., 1996), or dehydration-induced phase transitions of the phospholipid membranes (Webb et al., 1996). It

was observed that COR6.6 neither reduced nor prevented these forms of damage suggesting that this peptide does not have a cryoprotective effect on membranes *in vitro*.

The second theory that has evolved to explain cryoprotection involves the stabilization of peptides through a mechanism of preferential exclusion, which leads to preferential hydration and stabilization of proteins (Carpenter and Crowe, 1988). Since some free amino acids have been shown to have cryoprotective properties, it was suggested that cryoprotective ability of a peptide is independent of secondary structure (Carpenter and Crowe, 1988). Similarly, polypeptides with desiccation protection properties are also suggested to function independent of structure (Asghar et al., 1994). A highly unstructured polypeptide, such as BN28 which is cytoplasmically localized, could have cryoprotective properties. Therefore, BN28 may be involved in the stabilization of cellular proteins in the event of extracellular freezing.

To address the possible role of BN28 as a cryoprotectant, an *in vitro* assay that tested the principle of preferential exclusion was utilized (Carpenter and Crowe, 1988). In this assay, the ability of compounds to prevent denaturation of the freeze-labile enzyme LDH was determined following a freeze-thaw stress. This assay was previously used to determine the cyoprotective properties of both COR15 in *Arabidopsis thaliana* (Lin and Thomashow, 1992a). In both instances, the peptides were capable of preventing the freeze-denaturation of LDH at significantly lower concentrations than other tested compounds.

In the present study, rBN28 affords virtually 100% protection from freeze-denaturation over the physiological concentration range of BN28 (Fig.12, A) and was shown to be a more effective cryoprotectant than sucrose, proline and BSA (Table 3). Therefore, in this assay, BN28 does appear to have cryoprotective properties.

To determine whether the source of the enzyme used in the assay alters the cryoprotective properties of rBN28, the effectiveness of rBN28 to protect a freeze labile enzyme found in plants cell was addressed. Over 80% G6PDH activity was retained at the physiological concentration for BN28. This level was well above the 15% residual G6PDH activity when frozen in the absence of rBN28 (Fig. 12, B). The effectiveness of rBN28 as a cryoprotectant for G6PDH, another freeze-labile protein (Tamiya et al., 1985) shows that cryoprotection is independent of the source of the enzyme and suggests that protection is dependent on the properties of the cryoprotectant tested.

Critics of this cryoprotection assay suggest that the *in vitro* freezing of a protein in solution does not mimic the *in situ* freezing-induced dehydrative stress. During tissue freezing there is a loss of cytoplasmic water which leads to an increase in salt concentration in the cell. This, in turn, interferes with hydrogen bonding, altering protein structure and function. Similarly, the freezing of a protein in solution leads to the partitioning of salts away from the forming ice. This again leads to an increase in salt concentration, which disrupts protein structure and function. *In vitro* freezing in solution does mimic the freezing-induced dehydrative stress experienced by a

peptide and therefore, this assay can be used as a model for enzyme inactivation *in situ*.

On the basis of the above, it was determined that in this *in vitro* assay, an unstructured protein, rBN28, has cryoprotective function in solution. Although other low temperature-inducible proteins have been shown to have cryoprotective properties (Lin and Thomashow, 1992a; Houde et al., 1995), this is the first time that an amorphous protein, in plants or animals, has been shown to have cryoprotective behavior.

While these results are important in terms of understanding the relationship between structure and function, cryoprotection does not preclude a possible second role for BN28 *in situ*. To address this possibility, rBN28 and BN28 were characterized biochemically.

To begin, comparisons on native and urea gels were carried out to elucidate multiple forms of BN28 which possibly exist within the plant. Both non-denaturing native and urea PAGE showed multiple forms of BN28 while rBN28 appeared as a single peptide with slightly greater mobility (Fig. 13). Both native and urea gel systems separate proteins on a charge to mass ratio. However, urea gels further dissociate multimeric complexes. The similar results between the two gels suggest that the multiple bands observed are not quaternary structure but rather may result from post-translational modifications of BN28 peptide *in vivo*. These modifications might include changes to both mass and charge of the peptide.

To determine possible changes in the charge of BN28, isoelectric focusing of both rBN28 and BN28 was carried out. It was

shown that the pI of BN28 is altered *in vivo* (Fig. 17). Therefore, post-translational modifications have altered the charge substantially from the predicted value. Further analysis using 2-D gels and Western blotting revealed that BN28 underwent alterations in both size and charge (Fig. 18). 2-D gels of recombinant and native forms of COR6.6 also showed differences in migration patterns, however, these differences were not further investigated (Gilmour et al., 1996).

The presence of several BN28 bands on native and urea gels may also be explained by a small multi-gene family. Kurkela and Borg-Franck (1992) showed that a multi-gene family exists in *Arabidopsis thaliana* encoding both KIN1 and KIN2. Since the migration of the single band observed for rBN28 (on both native and urea PAGE gels) does not correspond to any of the 3 bands for BN28, it is felt that post-translational modifications to rBN28 are occurring altering the relative mobility of the nascent peptide into 1 or more of the 3 BN28 bands *in vivo*. The results of the urea and native gels, together with the IEF and 2-D gels strongly suggest that post-translational modifications occur to BN28 altering both size and charge.

Post-translational modifications of proteins, altering both size and charge, include protein phosphorylation (Datta and Cashmore, 1989; Felix et al., 1991; Sarokin and Chua, 1992; Daminov et al., 1992) and glycosylation (Hames and Rickwood, 1990). Monroy et al., (1993) observed that phosphorylation of low temperature-inducible proteins played an important role in the acquisition of freezing tolerance in *Medicago sativa*.

To address the possible phosphorylation of BN28, the amino acid sequence was analyzed for putative phosphorylation sites. It was observed that the protein contained 7 possible phosphorylation sites (Fig.16). Phosphorylation at various combinations of these sites could account for the observed differences in size and charge of BN28. However, initial attempts at *in vivo* phosphorylation of leaf proteins were inconclusive and therefore were not presented.

Evidence exists in literature showing that some low temperature-inducible proteins are glycosylated (Sieg et al., 1996). The alterations in both size and charge to BN28 could occur through the addition of sugar residues (Hames and Rickwood, 1990). Therefore, glycosylation of BN28 was addressed. Amino acid sequence analysis showed that BN28 contains 11 putative glycosylation sites (Fig. 16). The use of a carbohydrate specific stain to detect glycoproteins in acclimated leaf proteins did not identify BN28 as a glycoprotein (Fig. 19). Prior to analysis it was observed that the cellular concentration of BN28 (Boothe et al, 1995) was at a level not detectable by the glycoprotein stain. Therefore, the protein sample was concentrated through boiling to remove extraneous peptides and then acetone precipitation. Such treatment, although severe, would not effect the sugar residues of glycoproteins. This is supported by the fact that boiling did not alter BN28 migration on native and urea gels (Fig. 13). The removal of sugar residues from proteins is carried out enzymatically or through severe chemical treatment for several hours (Sambrook et al., 1989). Therefore, acetone precipitation would not remove sugar residues from polypeptides. These facts strongly suggest that the conditions for

protein concentration would not invalidate the observed results for glycoprotein detection. Therefore, I feel that BN28 is not a glycoprotein.

Although it was shown that BN28 undergoes post-translational modifications in size and charge, no evidence exists in literature to suggest that these kinds of changes would alter the cryoprotective or antifreeze properties of peptides. Therefore, the modified BN28 will apparently still exhibit cryoprotective activity.

Knowledge of the cryoprotective properties of rBN28 and the post-translational modifications to the protein does not provide insight into a possible role for BN28 in the acquisition of freezing tolerance during low temperature acclimation. This was addressed by determining the spatial and temporal accumulation of BN28 in acclimating seedlings.

Although spatial variations of such proteins have been studied (Perras and Sarhan, 1989), the relationship between the accumulation of low temperature-inducible proteins and the developmental stage of the plant or organ have not been addressed. Acclimation-induced changes in leaf dry weight, chlorophyll, carotenoids and benzoquinones have been shown to differ depending on the developmental stage of the leaf at the time of low temperature-exposure (Krol and Huner, 1984). Young leaves are also more actively dividing during acclimation with divisions in leaf one approximately 50% less than divisions in leaf four (Singh, 1995).

BN28 accumulates differently in leaves at different developmental stages (Fig. 25). In plants at the four and seven leaf stages, BN28 accumulates to the highest levels in the youngest

leaves, becoming progressively less with increased leaf age (Fig. 25). Similar patterns of BN28 accumulation in plants at the four and seven leaf stage suggest that accumulation occurs independent of plant growth stage, but dependent upon tissue development. High levels of BN28 accumulation in apical meristem tissue, which becomes undetectable in more developed stem tissue (Fig. 23), further supports a relationship between tissue development and the accumulation of BN28.

Seedlings at the first leaf stage show limited accumulation of BN28 in the youngest leaf. Although this appears to contradict the previous statement regarding the high accumulation of BN28 in the youngest leaf, it has been shown that very young seedlings acclimate to a lesser degree than older ones (Peltier, 1932; Paquin and Pelletier, 1987; Cloutier et al., 1990). Therefore, the lack of BN28 accumulation in seedlings at the first leaf stage may be related to the early developmental stage of the seedlings and their inability to acclimate.

Low BN28 levels in mature leaves are not attributed to senescence. It has been shown that chlorophyll a/b ratios decrease in leaf tissue during senescence (Back and Richmond, 1971; Gepstein, 1979; Jenkins and Woolhouse, 1991). No significant difference in chlorophyll a/b ratios were found between young and mature leaves, thereby indicating that the differences in accumulation of BN28 were probably not caused by senescence (Fig. 28).

To further address the relationship between developing tissue and accumulation of BN28, Northern and Western blots were utilized. Comparisons between *bn*28 message (Fig. 27) and BN28 protein (Fig.

25) in young and old leaf tissue of acclimated seedlings revealed that the disappearance of BN28 in older tissue was not correlated with a similar disappearance of message. This discrepancy suggested that the differences observed the in accumulation of BN28 in leaves at different developmental stages are regulated at the post-transcriptional level.

The results of the present study were also used to gain insight into whether BN28 was involved in the acquisition of freezing tolerance. Numerous studies have revealed a correlation between the synthesis of specific proteins and increased freezing tolerance (Mohapatra et al. 1987b; Gilmour et al. 1988; Lång et al., 1989). However, it is not clear if these peptides are involved in the acquisition of freezing tolerance. Guy and Haskell (1987) showed a strong correlation between freezing tolerance and synthesis of cold acclimation inducible proteins during acclimation and deacclimation suggesting that these peptides probably play a role in the acquisition of freezing tolerance. However, for most other low temperature-inducible proteins, the role in acclimation remains unknown.

Previously, it was concluded that BN28 does not provide protection from freezing damage (Boothe et al., 1995). This relationship was re-examined in this study. The acquisition of freezing tolerance, as measured by ion leakage (Fig. 20), paralleled the accumulation of BN28 (Fig. 21). However, during deacclimation, the leaf maintained a substantial amount of freezing tolerance (Fig. 20) while BN28 disappeared (Fig. 21). When one considers that there are numerous proteins associated with freezing tolerance and each is probably contributing to the overall tolerance, it becomes obvious

that the loss of one polypeptide should not lead to a total loss of freezing tolerance. This, therefore, supports the hypothesis that BN28 confers freezing tolerance. Further evidence for a role in freezing tolerance arises from the fact that differences in BN28 accumulation between young and mature leaves (Fig. 25), are also closely related to leaf freezing tolerance (Fig. 26). High accumulations of BN28 were also found in tissues which are known to achieve a high degree of freezing tolerance (Fig. 22) (Perras and Sarhan, 1989; Andrews and Morrison, 1992). Finally, the strongest evidence comes from the cryoprotection assay. On the basis of these results, it is proposed that BN28 has a role in the acquisition of freezing tolerance in *Brassica napus*.

BN28 may also have a role in metabolic adjustment to low temperature growth. Immunohistochemical localization comparing the emerging leaf and leaf 4 suggested that as the emerging leaf grew and developed, BN28 accumulation decreased in the epidermal layer (Fig. 30). However, BN28 remained detectable in the spongy mesophyll and guard cells. The literature suggests that acclimation occurs differently in guard and mesophyll cells of the arctic plant *Saxifragara cernua* L. (Mawson and Cummings; 1989, 1991), with the guard cells maintaining higher quantum yields than is measured in mesophyll chloroplasts when acclimated at 10°C. This again supports the claim that BN28 is accumulating in tissues and cells that are metabolically active to possibly enhance the freezing tolerance in specific cell types. Further evidence to support this comes from the result that BN28 is greatly reduced in more

developed stem away from the apical meristem (Fig. 23) and by a loss of BN28 in eppidermal cells of more developed leaves (Fig. 30).

BN28 was shown to be induced by low temperature only and not by ABA, drought, and salinity (Fig. 34). The fact that BN28 is not induced by salinity and drought suggests that BN28 does not play a direct role in freeze-induced dehydrative stress. This again would support a possible role for BN28 in the metabolic adjustment of plant tissue to growth at low temperature.

Summary

The goal of the present physiological, biophysical and biochemical analyses was to test three hypotheses that have appeared in literature. The first proposed that the *kin* gene family (of which *bn*28 is a member) encode antifreeze proteins, based on the predicted amino acid sequence and predicted α–helical conformation (Kurkela and Franck, 1990). The second hypothesis proposed that relatively unstructured proteins could protect proteins from denaturation during dehydrative stress (Asghar et al., 1994)(a form of injury incurred as a result of freezing). The third hypothesis proposed that BN28, like the CAP proteins (Guy and Haskell, 1987), is involved in the acquisition of freezing tolerance.

The results strongly suggested that BN28 is not an antifreeze protein. It was demonstrated, for the first time, that an amorphous protein could display cryoprotective behavior (within cellular concentrations). This provides a crucial link between function and experimentally determined structure.

The present study then focused on results obtained from experiments utilizing rBN28 to determine if they could provide insight into the role of BN28 *in situ*. Results from this portion of the work showed that BN28 and rBN28 differ significantly. It was shown that BN28 undergoes post-translational modifications into multiple forms with altered size and charge. The implications of this on cryoprotective function are not known. It is understood that these changes will not impart antifreeze activity on the protein. Further work on this must be carried out.

To address these concerns BN28 first needs to be purified from acclimated seedlings through a combination of various chrmatographic techniques. Once this is completed, cryoprotection, structure and post-translational modifications need to be examined. If it appears that BN28 is involved with the metabolic adjustment to low temperature growth, then protein/protein interactions and possible protein/nucleic acid interactions should be addressed.

A cryoprotective function does not preclude another function. As such, the spatial and temporal accumulation of BN28 was examined in order to determine whether BN28 might confer freezing tolerance or be involved in the metabolic adjustment to low temperature growth. Evidence from several experiments *in situ* suggested that BN28 plays a role in the acquisition of freezing tolerance.

In conclusion, this study has characterized a low temperature-induced protein from *Brassica napus*. At the onset of this work, no other group had reported gaining an understanding of function for low temperature inducible proteins, with the exception of cursory examinations of predicted structure and cryoprotection assays. Although unable to definitively ascribe a role to BN28, the results presented have been successful in showing that BN28 is not an antifreeze protein and that it has cryoprotective behaviour. This and the *in situ* results support a role in the acquisition of freezing tolerance. Results from both the secondary structure determinations and cryoprotective properties of rBN28 strongly suggest a possible link between protein structure and function, which have laid the groundwork for further study of the native protein.

Fig. 2. Transformation vector for bacterial expression system. The pMAL-c plasmid contains the *bn*28 cDNA, the gene encoding the maltose binding protein (malE) and a factor Xa protease recognition site(Xa). The MBP-*bn*28 fusion gene is regulated by a IPTG inducible promoter (P_{tac}), and contains a gene conferring ampicillin resistance (Amp^r) and a gene for β-galactosidase (lac Zα).

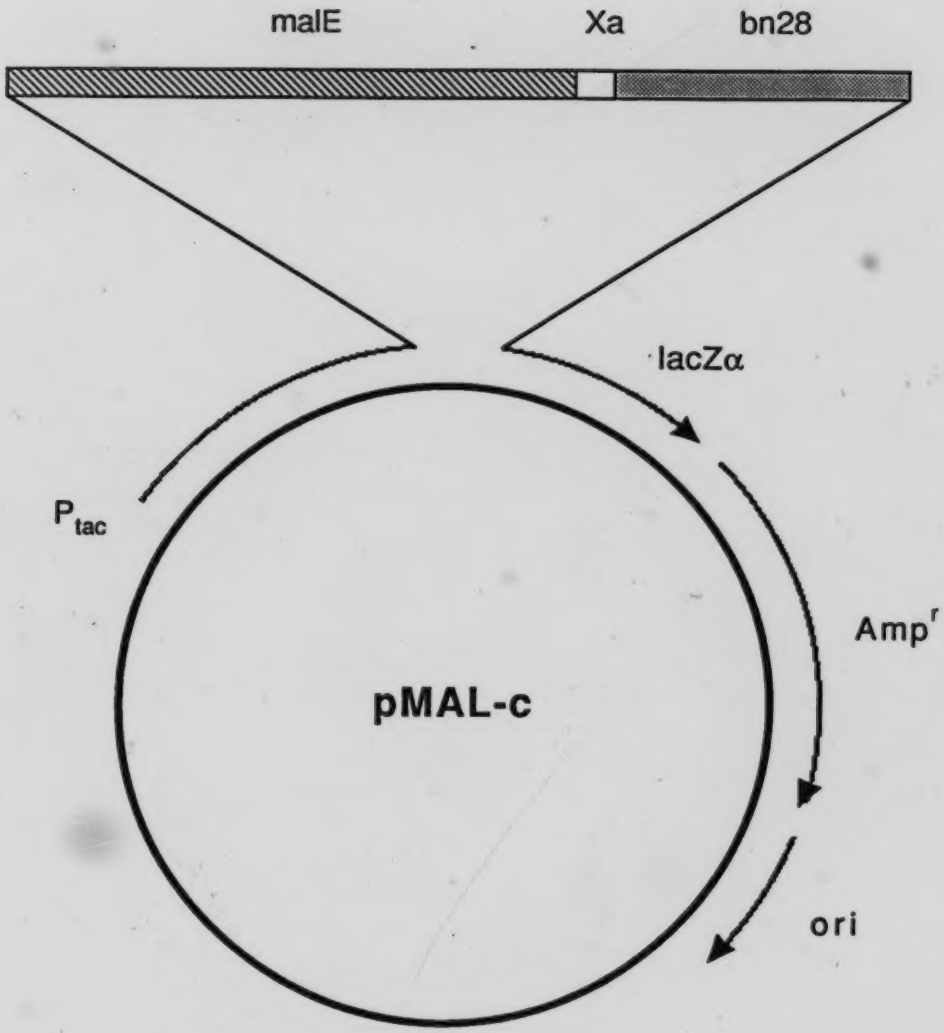

malE
Xa
bn28
lacZα
P tac
Amp r
pMAL-c
ori

Fig. 3. Coomassie stained SDS-PAGE gel of proteins from *E.coli* transformed with the p-MAL-c expression vector containing the *bn*28 cDNA. Lane c is SDS-soluble protein from non-induced *E. coli.* Lanes 1, 2, 3 represents protein from *E. coli* induced with 0.3 mM IPTG for 1, 2, and 3 hours, respectively. The induced fusion protein appeared as a 45 kDa peptide. Lanes 4, 5, and 6 represent protein from *E. coli* treated for 3 hours with 0.1 mM, 0.2 mM and 0.3 mM IPTG, respectively. All lanes were loaded with 60 μg of SDS-soluble protein. Molecular weights (kDa) are shown.

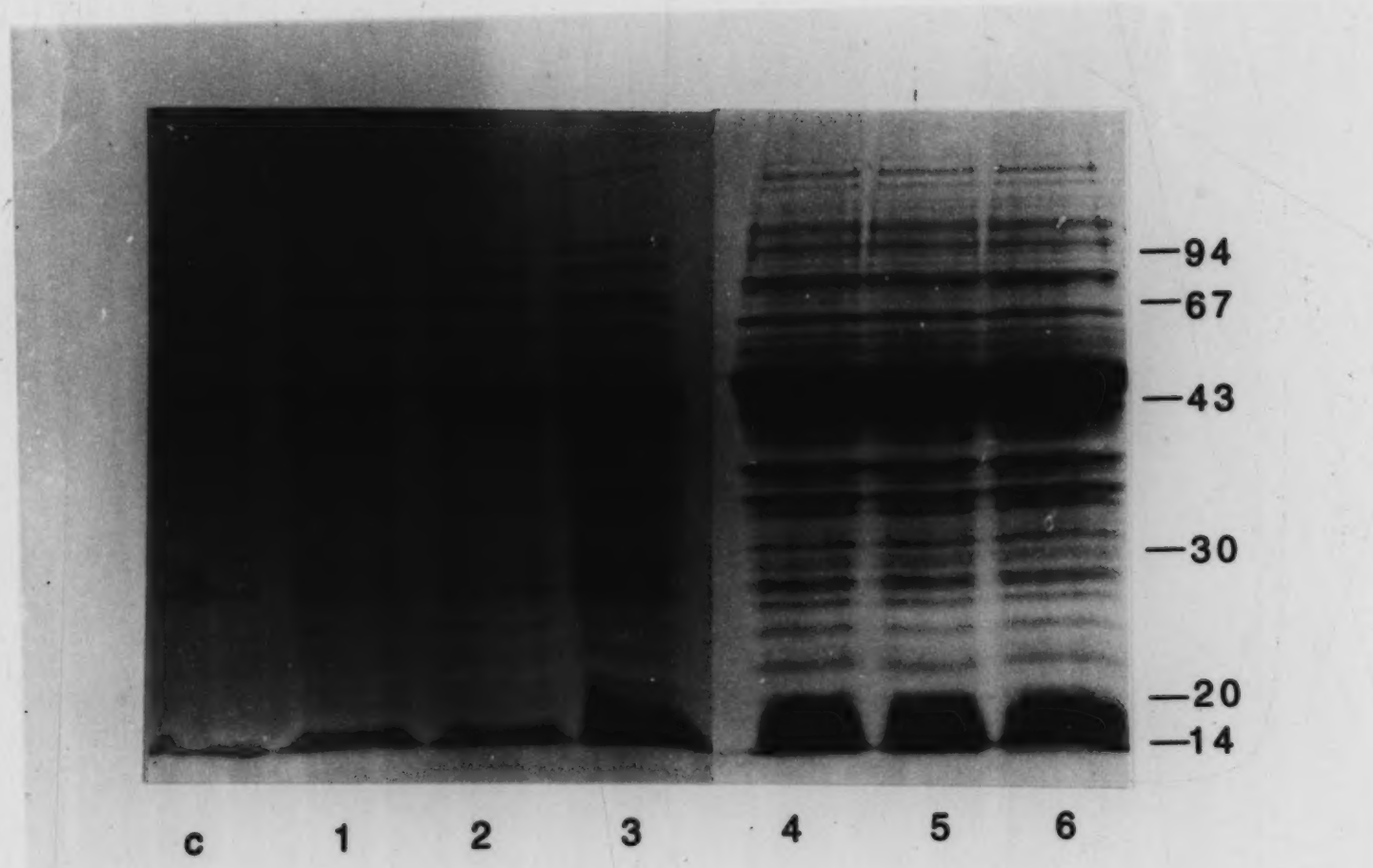

—94
—67
—43
—30
—20
—14
c
1
2
3
4
5
6

Fig. 4. Coomassie stained SDS-PAGE gel of proteins from *E.coli* transformed with the p-MAL-c expression vector containing the *bn*28 cDNA. Lane UI is SDS-soluble protein from non-induced *E. coli.* Lane I represents protein from *E. coli* induced with IPTG. The upper arrow indicates the 45 kDa MBP-BN28 fusion protein. Lane IC is protein from induced *E. coli* treated with factor Xa protease. The lower arrow indicates the 39 kDa MBP portion of the fusion protein. All lanes were loaded with 60 μg of SDS-soluble protein. Molecular weights (kDa) are shown.

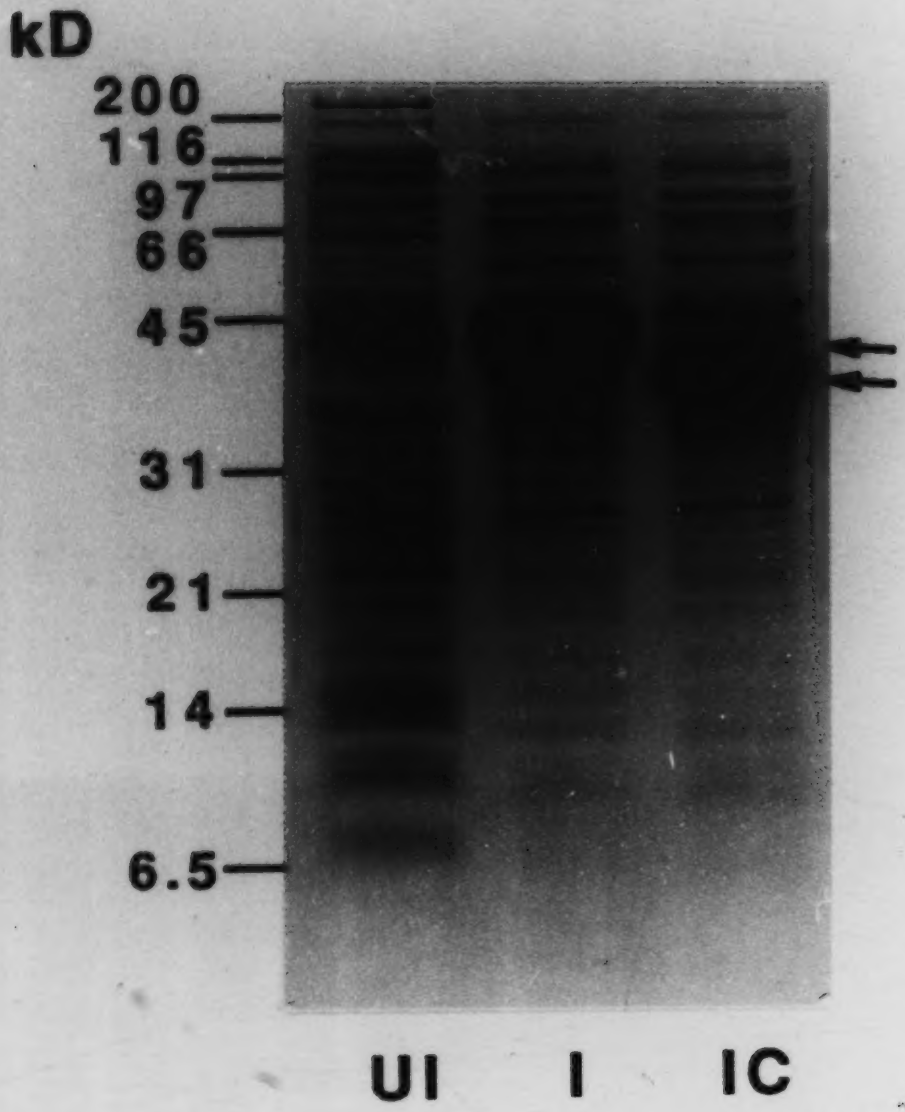

kD
200
116
97
66
45
31
21
14
6.5
UI
I
IC

Fig. 5. Silver stained SDS-PAGE gel of proteins from transformed *E.coli* containing the *bn*28 cDNA. Lane UI is SDS-soluble protein from non-induced *E. coli*. Lane I represents protein from *E. coli* induced with IPTG. The upper arrow indicates the 45 kDa MBP-BN28 fusion protein. Lane Ic is protein from induced *E. coli* treated with Factor Xa protease. The middle arrow indicates the 39 kDa MBP portion of the fusion protein. Lane P represents recombinant BN28 (rBN28) purified from other bacterial peptides by DEAE cellulose chromatography (30 ng). The bottom arrow indicates the 6.5 kDa rBN28. All other lanes were loaded with 30 μg SDS-soluble protein. Molecular weights are shown.

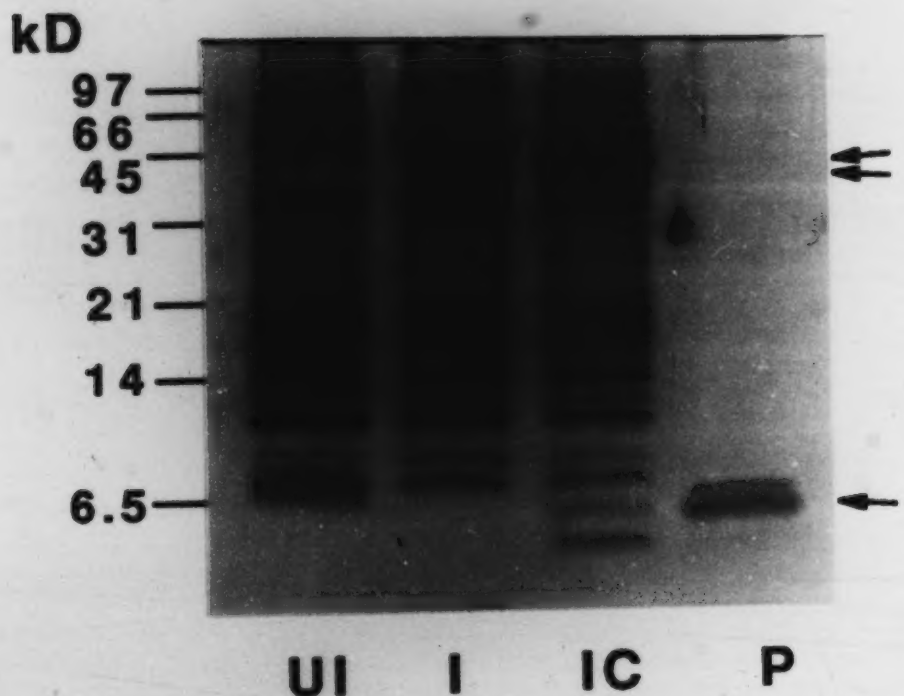

kD
97
66
45
31
21
14
6.5
UI
I
IC
P

Fig. 6. Coomassie stained SDS-PAGE gel of proteins collected during attempts to purify MBP/rBN28 using amylose resin affinity chromatography. Lane 1 represents the unbound peptides that passed directly through the column. Lane 2 is the remainder of unbound peptides following two washes of the column. Lane 3 represents the peptides eluted from the column using maltose to competitively remove bound proteins. Lane 4 is a small sample of the amylose resin used to determine if any fusion protein remained bound to the resin following elution of bound peptides with maltose. This was repeated under low salt (LS) and high salt (HS) conditions. All lanes were loaded with 50 µl of the respective samples combined with 20 µl SDS loading buffer.

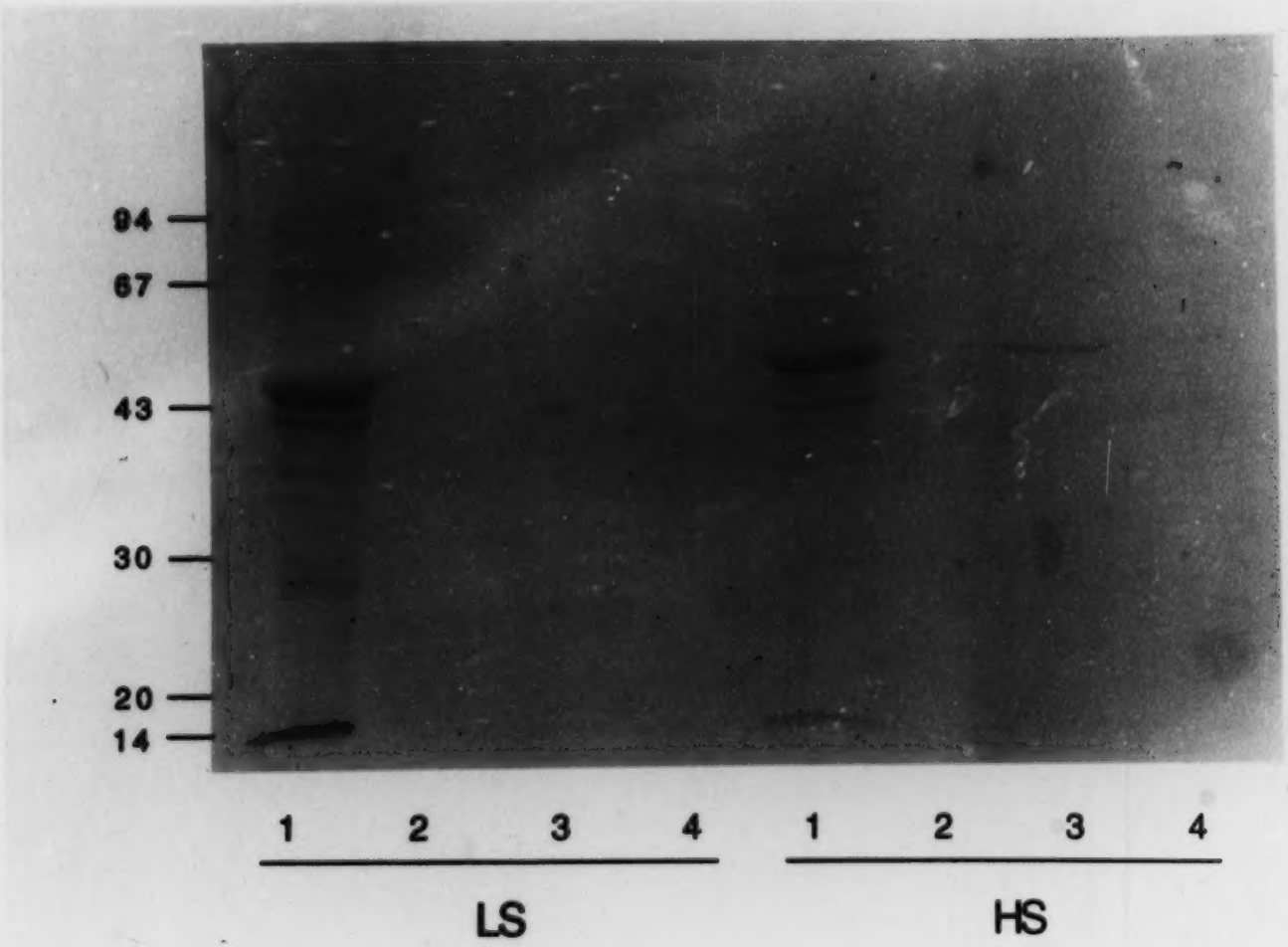

94
67
43
30
20
14
1
2
3
4
1
2
3
4
LS
HS

Fig. 7. Western blots of SDS-soluble proteins from leaf 4 of 42 day acclimated *Brassica napus* cv. Cascade seedlings at the four leaf stage. The blots were treated with pre immune serum (Pi), purified pre immune serum (PPi), immune serum (IgG) and purified immune serum (PIgG). All lanes were loaded with 80 µg SDS-soluble protein.

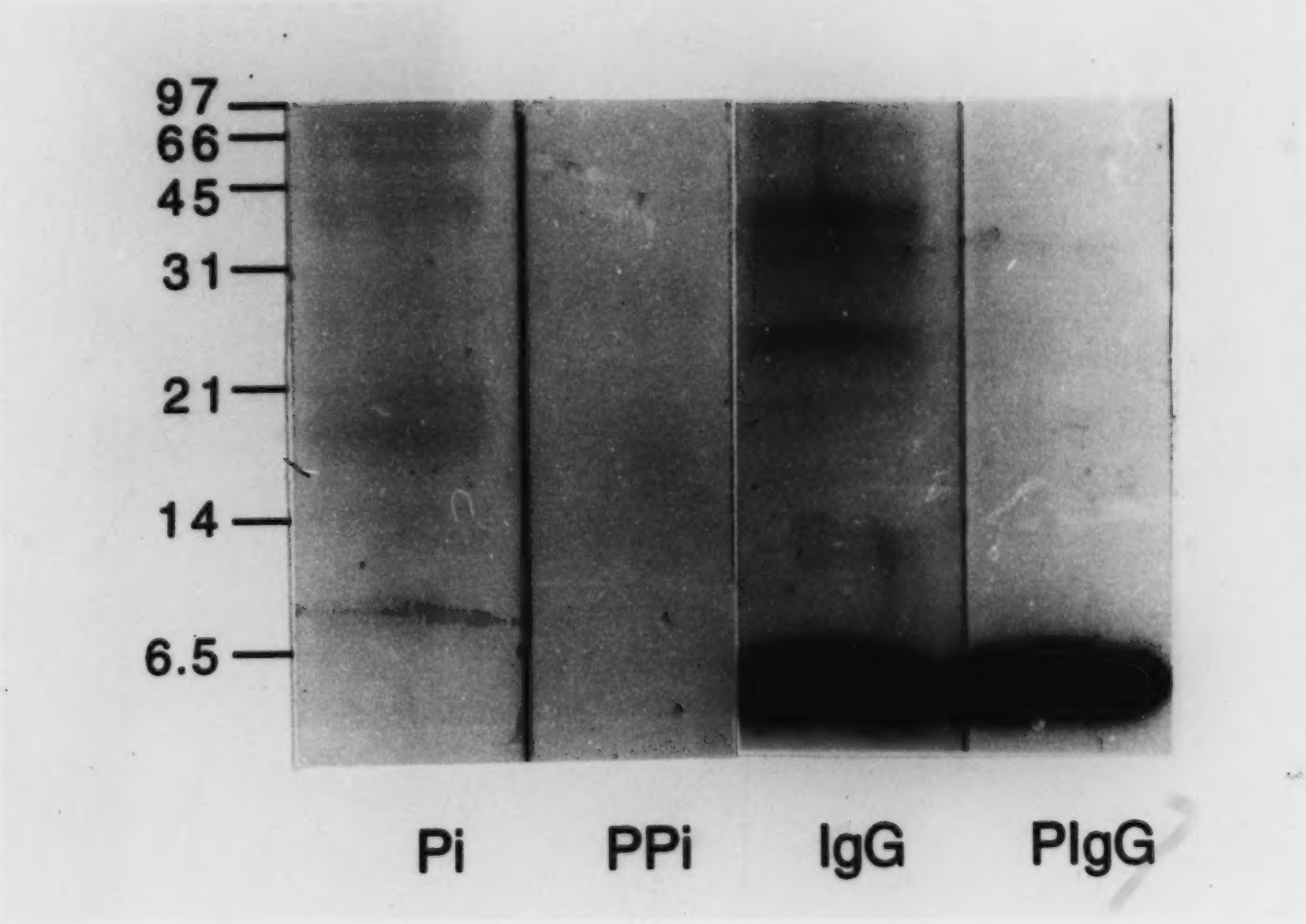
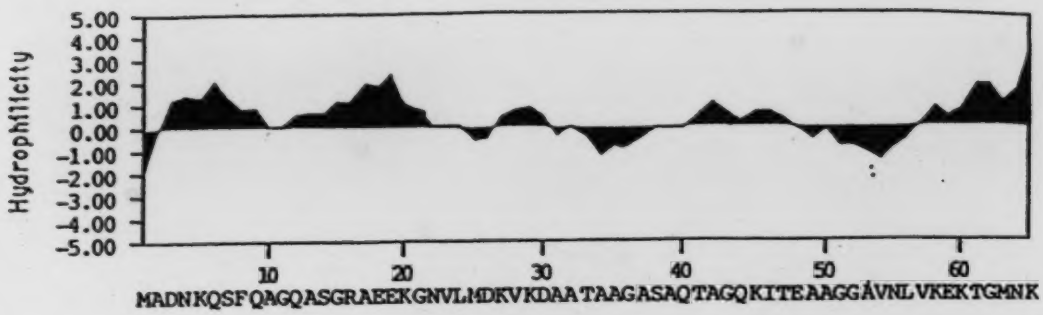

97
66
45
31
21
14
6.5
Pi
PPi
IgG
PIgG

Fig. 8. Hydrophilicity and secondary structure predictions for BN28. A) Hydropathic profile for BN28 determined according tothe algorithms of Kyte and Doolittle (1987). Positive values represent regions of hydrophilicity while negative values are hydrophobic.
B) Predicted secondary structure for BN28 based on the algorithms of Chou and Fasman (CF) (1978) and Robson and Garnier (RG) (1978) together with the concensus prediction (CFRG). Both the secondary structure and hydropathic profile were determined using a moving 7-residue window.

A


Hydrophilicity
5.00
4.00
3.00
2.00
1.00
0.00
-1.00
-2.00
-3.00
-4.00
-5.00
10
20
30
40
50
60
MADNKQSFQAGQASGRAEEKGNVLMDKVKDAATAAGASAQTAGQKITEAAGGAVNLVKEKTGMNK

B

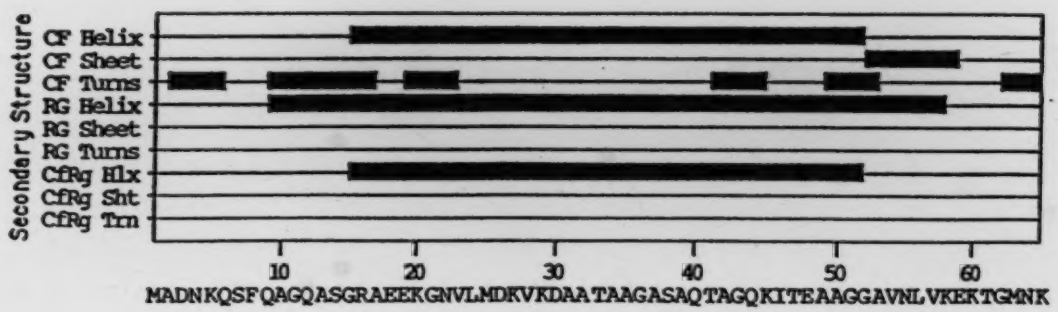

Secondary Structure
CF Helix
CF Sheet
CF Turns
RG Helix
RG Sheet
RG Turns
CfRg Hlx
CfRg Sht
CfRg Trn
10
20
30
40
50
60
MADNKQSFQAGQASGRAEEKGNVLMDKVKDAATAAGASAQTAGQKITEAAGGAVNLVKEKTGMNK

Fig. 9 Circular dichroism spectra of rBN28 at 5°C. A) Spectra for rBN28. B) Changes in the spectra when varying amounts of trifluoroethanol (TFE) are added to rBN28 (v/v). (solid line=10 % TFE, dashed line=20 %, dash dotted line=30 %, dash-double dotted line=40 % and dotted line=50 % TFE). C) Spectra for rBN28 with the TFE removed by dialysis.

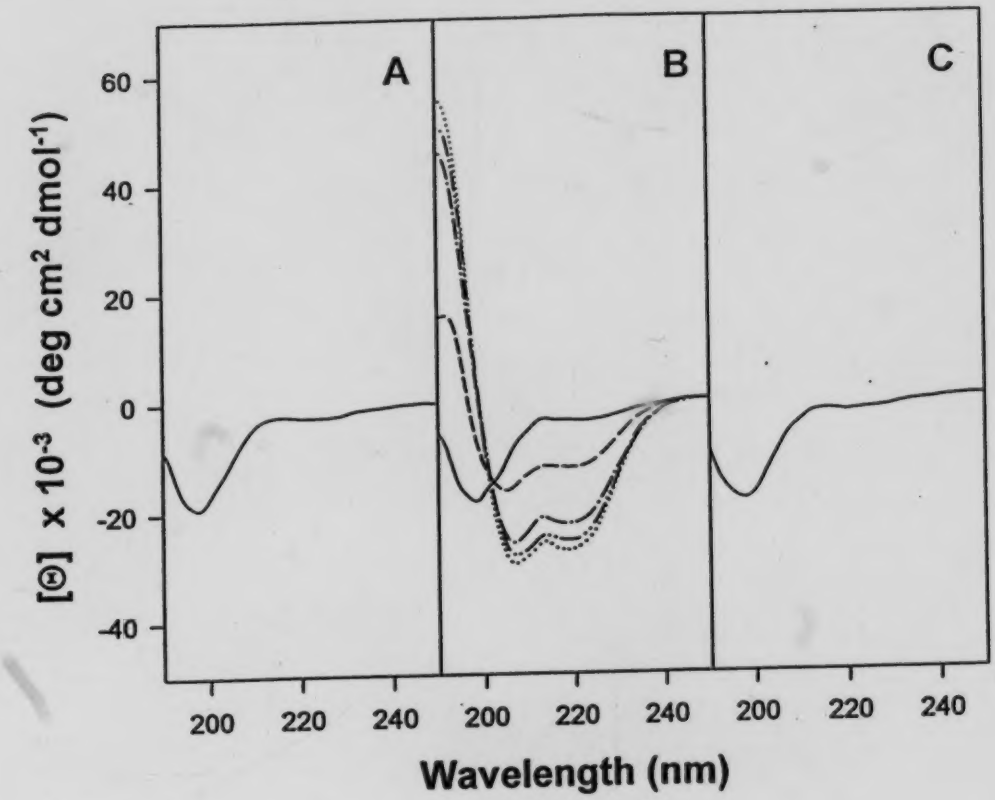

A
B
C
60
40
20
0
-20
-40
[Θ] x 10-3 (deg cm2 dmol-1)
200
220
240
200
220
240
200
220
240
Wavelength (nm)

Fig. 10. Content of unfrozen water in leaf 4 of 42 day acclimated *Brassica napus* cv. Cascade seedlings at the four leaf stage. Leaf tissue was exposed to +2.5°C to -17.5°C. Water values (LT) are expressed as grams of unfrozen water per gram dry weight of leaf tissue (g/g). These are averages of three replications. Analysis of variance found significat differences between +2.5°C and -5°C. (p=0.05).

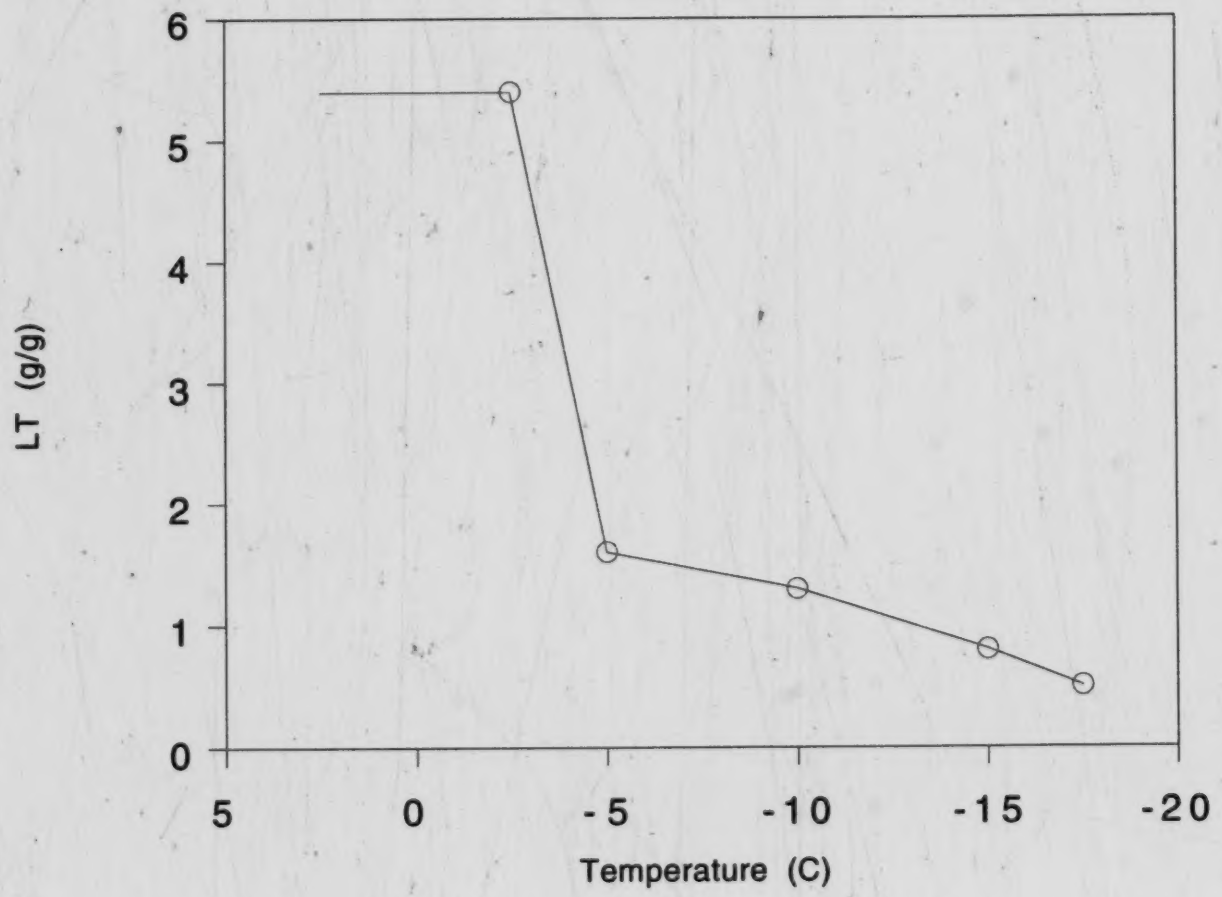

6
5
4
3
2
1
0
LT (g/g)
5
0
-5
-10
-15
-20
Temperature (C)

Fig. 11. Physiological range of BN28 concentrations (μM) in leaf 4 of 42 day acclimated *Brassica napus* cv. Cascade seedlings, at the four leaf stage, subjected over the temperature range +2.5°C to -17.5°C. These are averages of three replications. Analysis of variance found significat differences between +2.5°C and -5°C. (p=0.05).

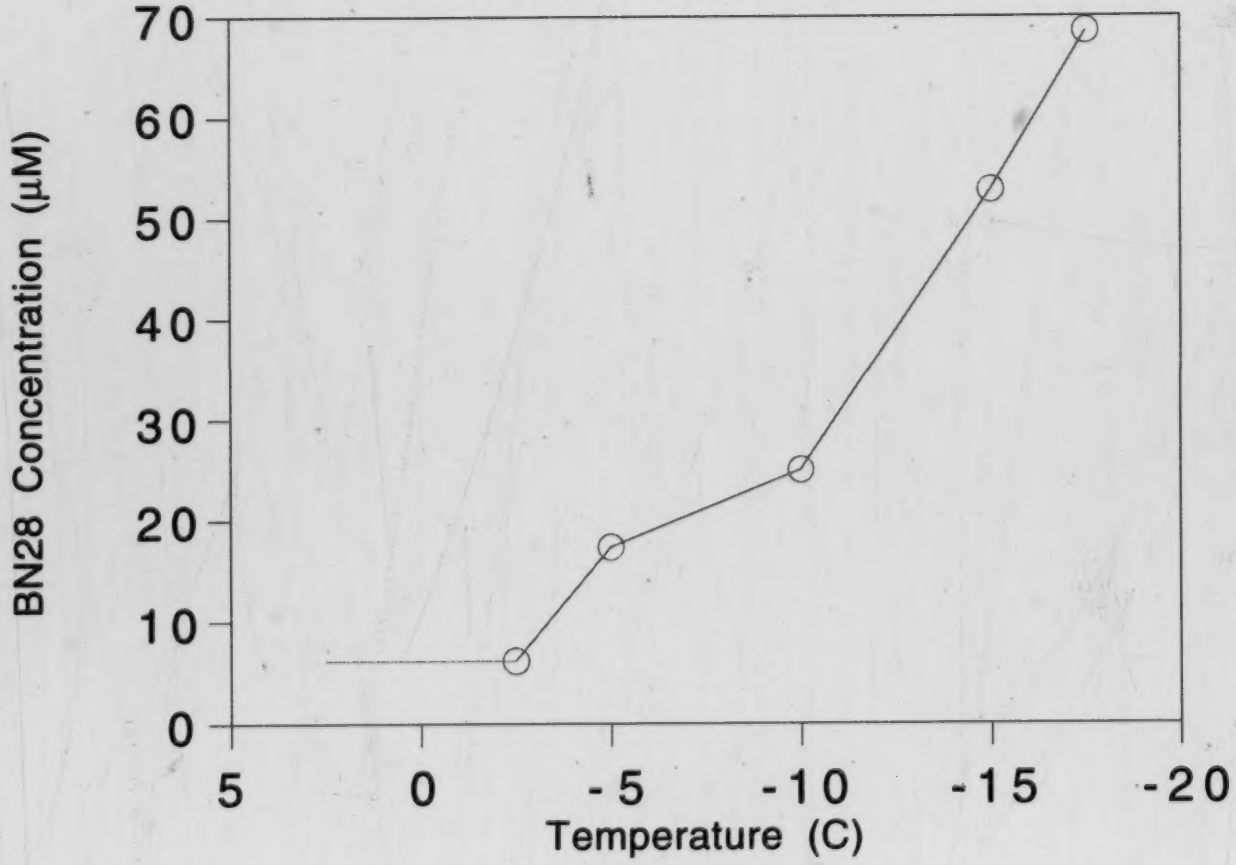
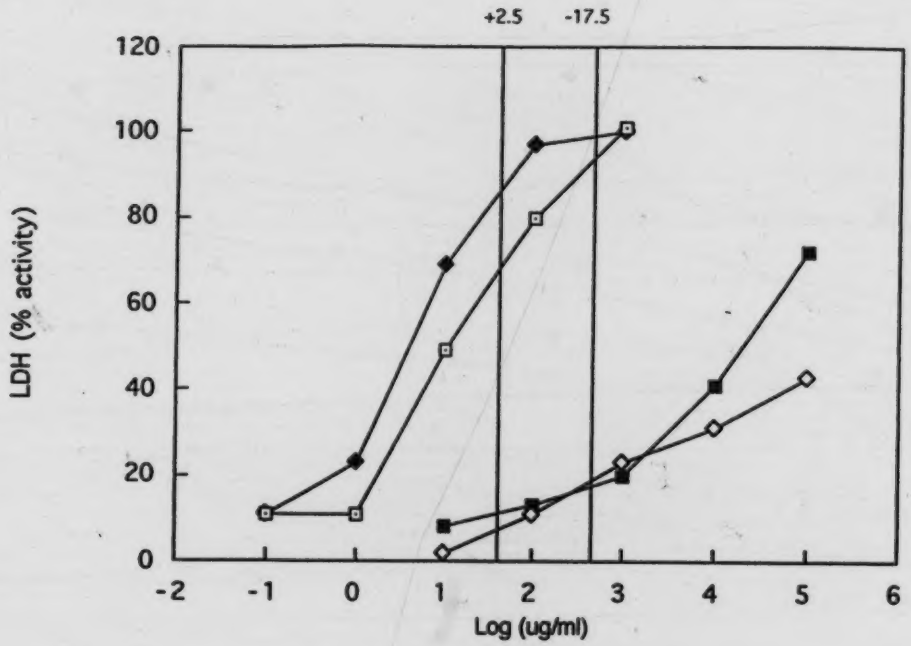

BN28 Concentration (µM)
70
60
50
40
30
20
10
0
5
0
-5
-10
-15
-20
Temperature (C)

Fig. 12. *In vitro* assays assessing cryoprotection of rBN28 relative to various compounds. A) Residual LDH activity following freezing (%) was plotted against log of solute concentration (µg/ml). Cryoprotection of rBN28 (closed diamonds) was compared with sucrose (open diamonds), proline (closed squares) and BSA (open squares). The two perpendicular lines represent the physiological range of BN28 concentrations previously determined at +2.5°C and -17.5°C. Analysis of variance showed no significant difference detween sucrose and proline. However, sucrose is significantly different from BSA and BN28, while BN28 is significantly different from BSA over the concentration range 1 to 100 µg/ml ($p=0.05$).

B) Residual G6PDH activity following freezing (%) was plotted against log of rBN28 concentration (µg/ml). The two perpendicular lines represent the physiological range of BN28 concentrations at +2.5°C and -17.5°C. All values are an average of three replicates.

A


+2.5
-17.5
120
100
80
60
40
20
0
LDH (% activity)
-2
-1
0
1
2
3
4
5
6
Log (ug/ml)

B

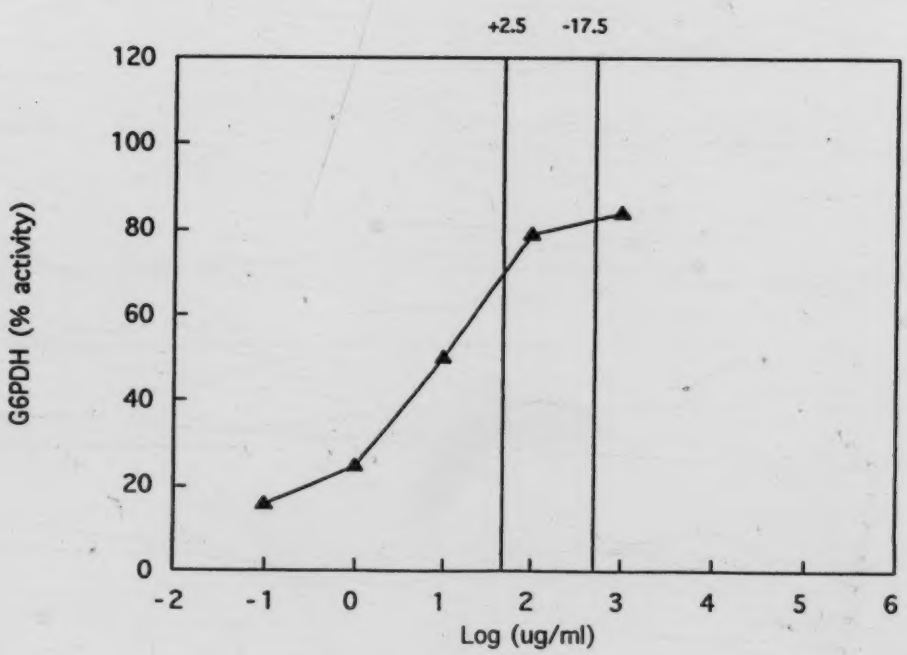
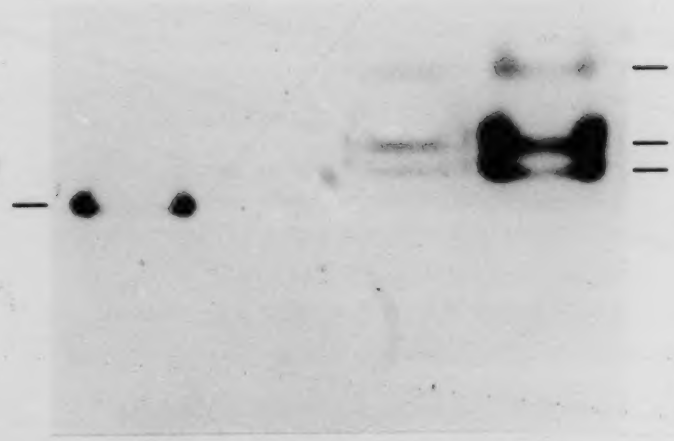

+2.5
-17.5
120
100
80
60
40
20
0
G6PDH (% activity)
-2
-1
0
1
2
3
4
5
6
Log (ug/ml)

Fig. 13. Western blots of proteins run on both a native (A), and urea (B) gel system. Lane rBN corresponds to purified rBN28, Na represents soluble protein from nonacclimated leaf tissue, A corresponds to protein from acclimated leaf tissue and Ab represents soluble protein from acclimated tissue that was boiled to concentrate the sample. All leaf samples were taken from leaf 4 of *Brassica napus* cv. Cascade seedlings at the four leaf stage. All lanes were loaded with 60 µg protein. Western blots were probed with anti-BN28 antibodies.

A



B

Fig. 14. Selectivity curve for a G50 Sephadex column used to analyse the relative size of BN28 and rBN28. Compounds with known molecular weights were fractionated through the column: a) aprotinin (6500 Da), b) rBN28 (6500Da), c) cytochrome C (12200 Da), d) ribonuclease A (13700 Da), e) lysozyme (14300 Da), f) chymotrypsinogin A (25000 Da). Their elution volumes (Ve) were combined with the total volume of the column (Vt=79.5 ml) and the void volume of the column (Vo=25 ml) to calculate the K_{avg} for the substance [K_{avg}=(Ve-Vo)/(Vt-Vo)]. The K_{avg} values were then plotted against the log of the molecular weight to obtain a selectivity curve showing the effective molecular weight range for protein separation.

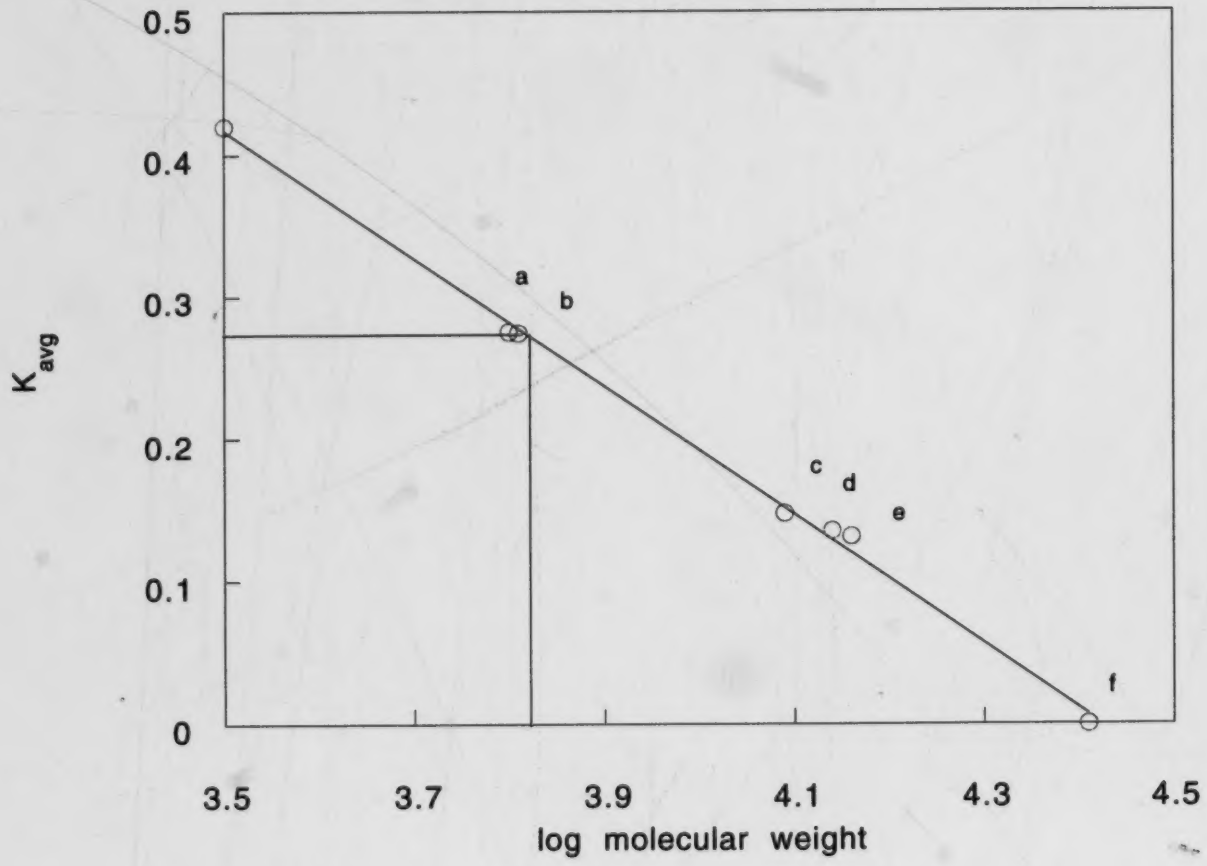

0.5
0.4
0.3
0.2
0.1
0
K_{avg}
a
b
c
d
e
f
3.5
3.7
3.9
4.1
4.3
4.5
log molecular weight

Fig. 15. Western blots of protein fractions collected from a G-50 Sephadex column and separated on SDS-PAGE. Upper blot are fractions from separation of purified rBN28. Lower blot are fractions from separation of BN28 extracted from leaf 4 of 42 day acclimated *Brassica napus* cv. Cascade seedlings at the four leaf stage. Each lane was loaded with 20 µl from each fraction. Western blots were probed with anti-BN28 antibodies. Lanes 1 through 7 represent fractions 11 through 17 following the void volume.

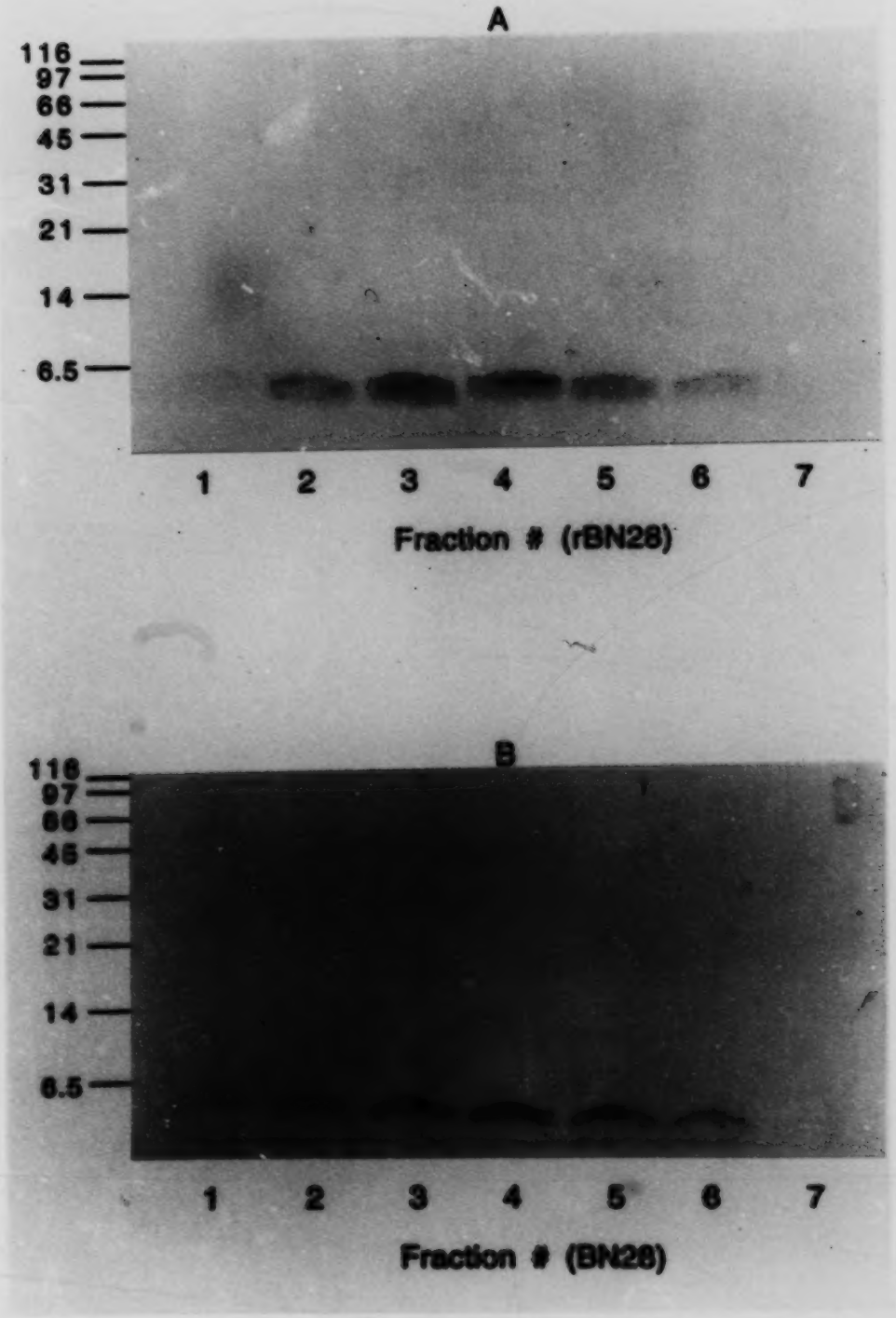

A
116
97
66
45
31
21
14
6.5
1 2 3 4 5 6 7
Fraction # (rBN28)
B
116
97
66
45
31
21
14
6.5
1 2 3 4 5 6 7
Fraction # (BN28)

Fig. 16. The predicted amino acid sequence for BN28 and COR6.6. Circled amino acids in BN28 represent putative phosphorylation sites_ while underlined amino acids represent putative glycosylation sites of BN28._

BN28 M-A D N K Q S F Q A G Q A S G R A E E K G N V L-M D K V

COR6.6 M S E T N K N A F Q A G Q A A G K A E E K S N V L-L D K A

BN28 K D A A T A A A G A S A Q T A G Q K I T E A A G G A V N L V K

COR6.6 K D A A A A A G A S A Q Q A G K S I S D A A V G G V N F V K

BN28 E K T G M N K

COR6.6 D K T G L N K

Fig. 17. Western blot of a one dimensional IEF gel. Soluble proteins were extracted from leaf 4 of 42 day acclimated *Brassica napus* cv. Cascade seedlings at the four leaf stage. Purified rBN28 (4.5 μg) and acclimated leaf protein (100μg) were focussed on a IEF gel (pI 3-10), capillary transferred to nitrocellulose and probed with anti-BN28 antibodies. pIs of standards are shown.

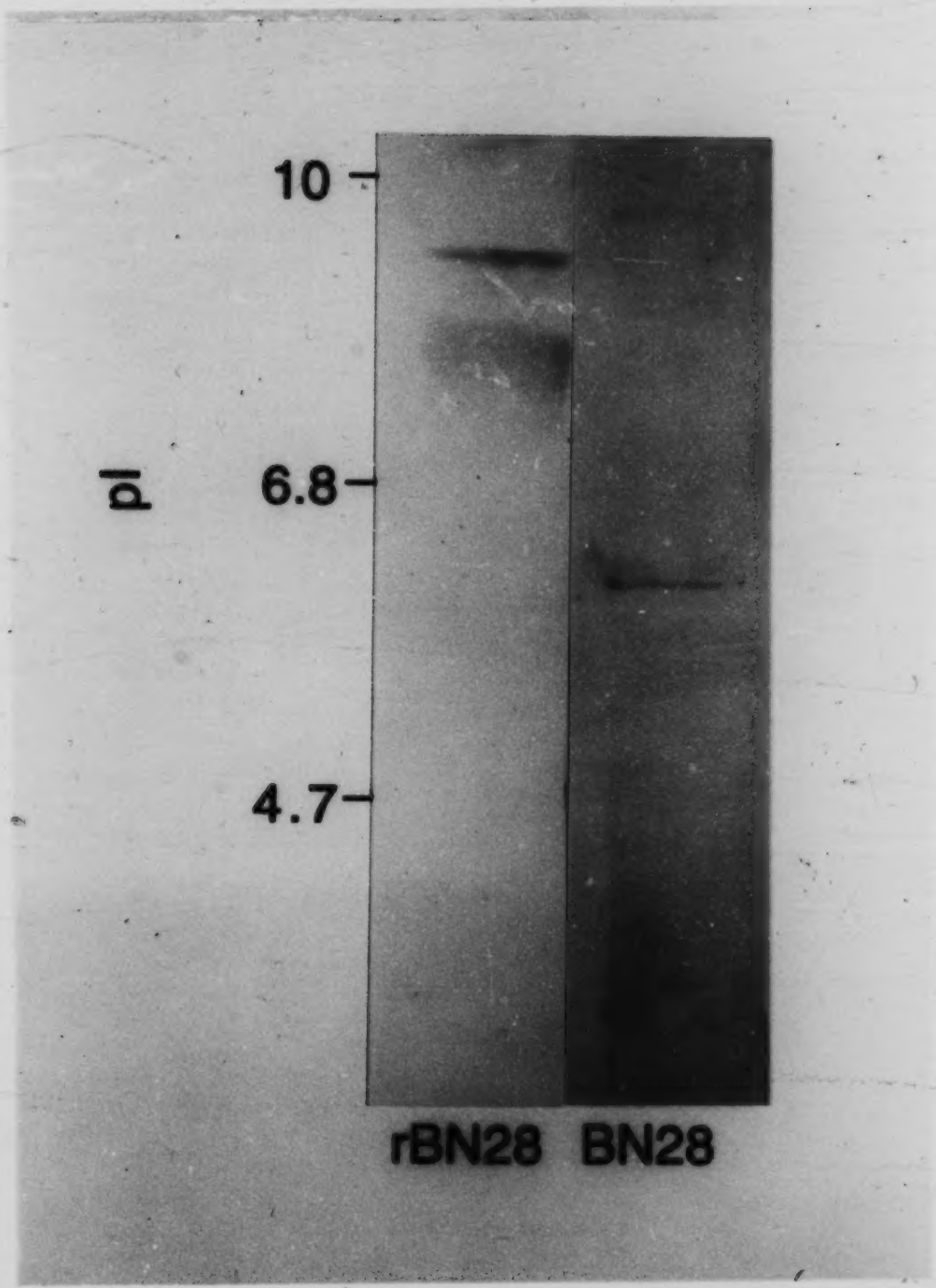

10
6.8
4.7
pI
rBN28
BN28

Fig. 18. Western blot of a two dimensional gel. Soluble proteins were extracted from leaf 4 of 42 day acclimated *Brassica napus* cv. Cascade seedlings at the four leaf stage. Acclimated leaf protein (400 µg) was focused on a tube IEF and then separated on SDS-PAGE in the second dimension. Proteins were transferred to nitrocellulose and probed with anti-BN28 antibodies. Molecular weight and pI standards are shown. Western blots were probed with anti-BN28 antibodies.

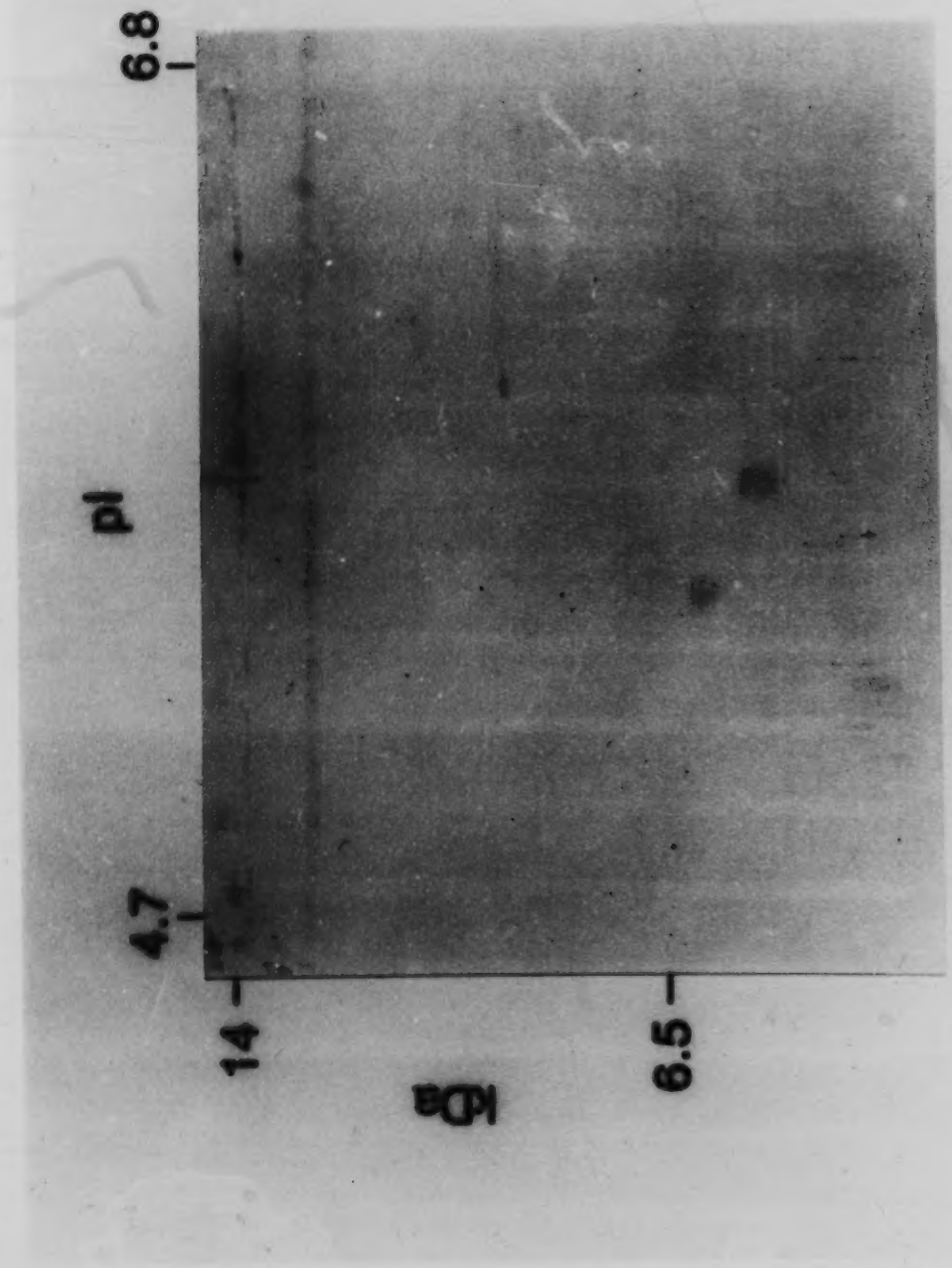

6.8
pI
4.7
14
kDa
6.5

Fig. 19. SDS-soluble proteins from 42 day acclimated *Brassica napus* cv. Cascade seedlings at the four leaf stage separated by SDS-PAGE. BN28 represents a Western blot of separated proteins (60 µg per lane). Gly represents a SDS-PAGE gel (30 µl of concentrated sample per lane) stained for glycopeptides. Samples stained for glycosolation were concentrated, by boiling and acetone precipitation, to increase the sensitivity of this procedure.

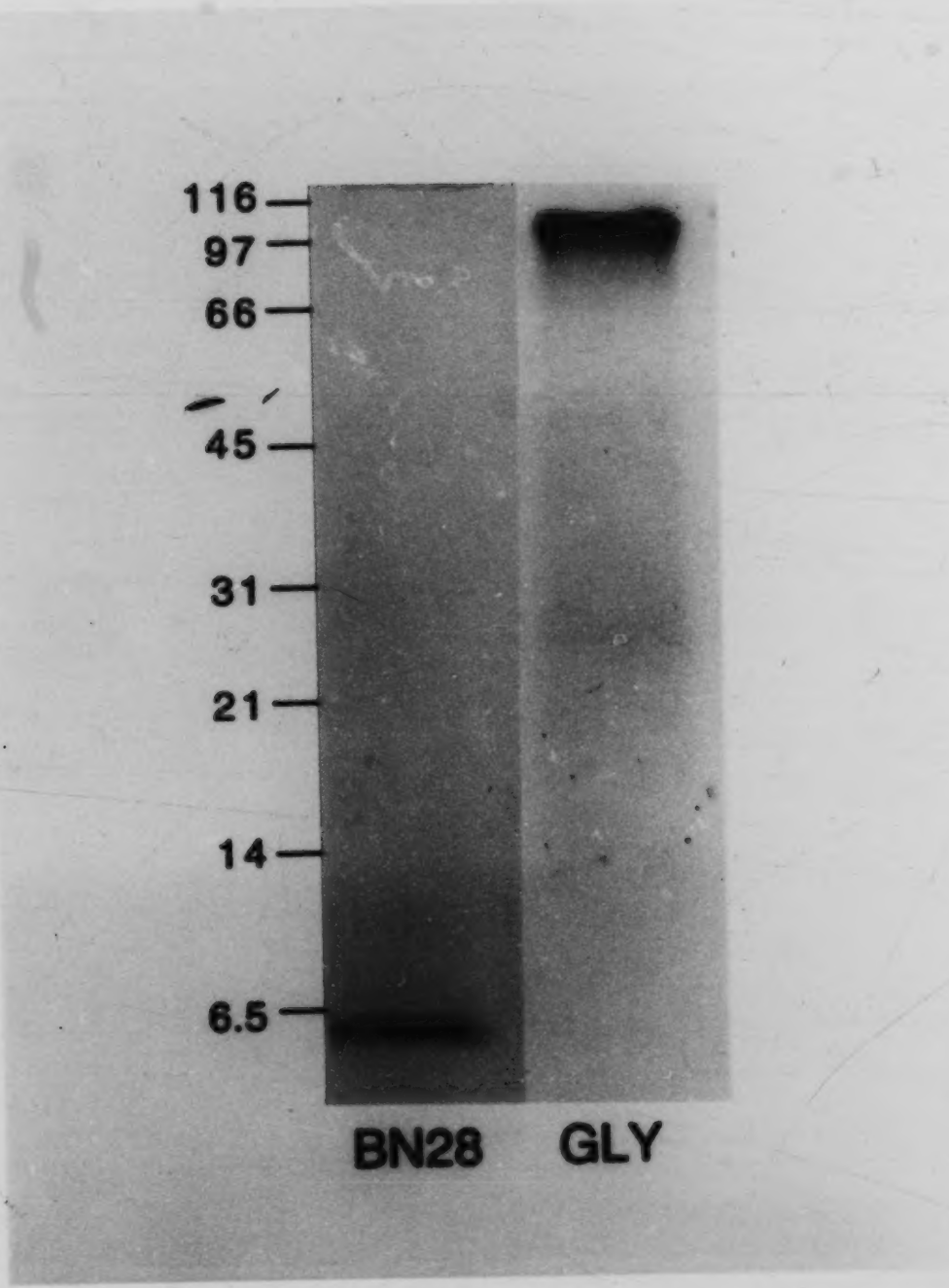

116
97
66
45
31
21
14
6.5
BN28
GLY

Fig. 20. Electrolyte leakage data for leaf 4 of *Brassica napus* cv. Cascade seedlings, at the 4 leaf stage, subjected to a freezing stress over the temperature range of 0°C to -17.5°C. Seedlings tested were either nonacclimated (NA), cold acclimated (CA) for 14, 28, 42 days or acclimated for 42 days then deacclimated (DA) for 2 and 7 days at control temperatures. Values represent the mean of three replicates.

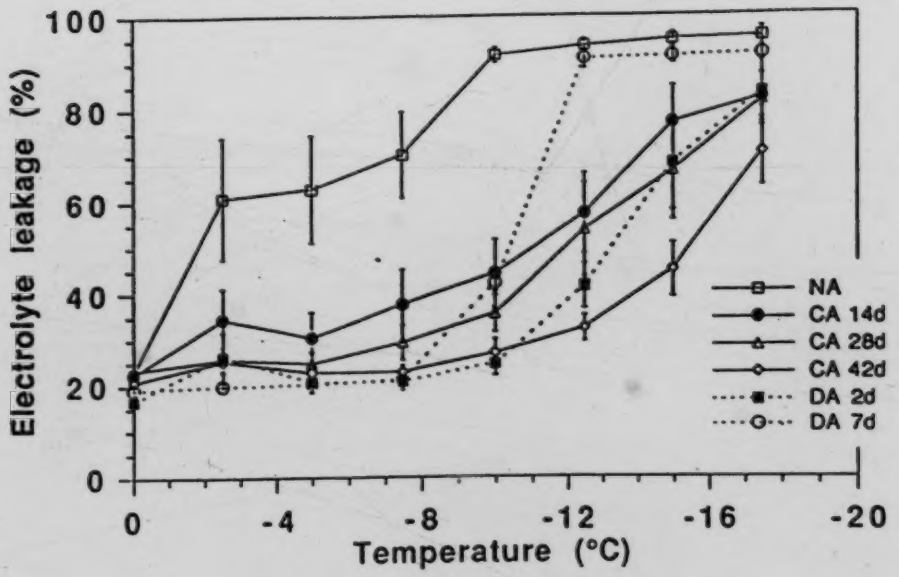
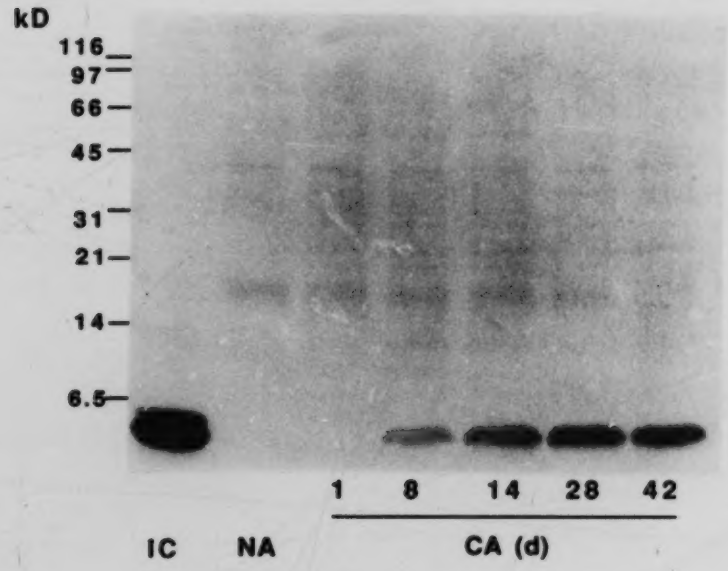

Electrolyte leakage (%)
100
80
60
40
20
0
0
-4
-8
-12
-16
-20
Temperature (°C)
NA
CA 14d
CA 28d
CA 42d
DA 2d
DA 7d

Fig. 21. Western blot of SDS-soluble proteins from leaf 4 from *Brassica napus* cv. Cascade seedlings at the 4 leaf stage. A) Nonacclimated (NA) and seedlings acclimated (CA) over 42 days (d). B) Seedlings were acclimated 42 days (d) and then deacclimated (DA) up to 7 days. Ic represents induced *E. coli* protein treated with factor Xa protease. All lanes were loaded with 60 µg SDS-soluble protein. Molecular weights are shown (kDa). Western blots were probed with anti-BN28 antibodies.

A


kD
116
97
66
45
31
21
14
6.5
1
8
14
28
42
IC
NA
CA (d)

B

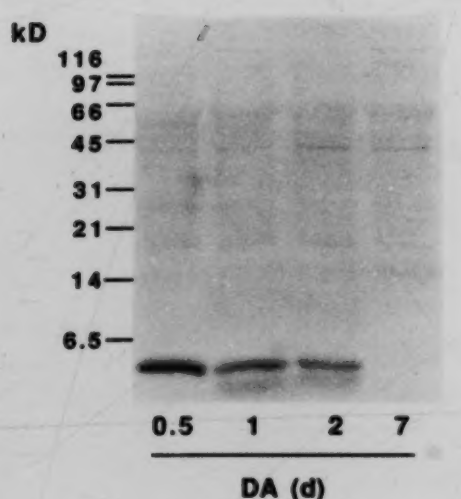

kD
116
97
66
45
31
21
14
6.5
0.5
1
2
7
DA (d)

Fig. 22. Western blots of SDS-soluble proteins extracted from leaf (L), root (R), apical meristem (Am), hypocotyl (H) and petiole (P), of control (c) and 21 day (21) acclimated *Brassica napus* cv. Cascade seedlings at the four leaf stage. All lanes were loaded with 60 μg protein. Western blots were probed with anti-BN28 antibodies.

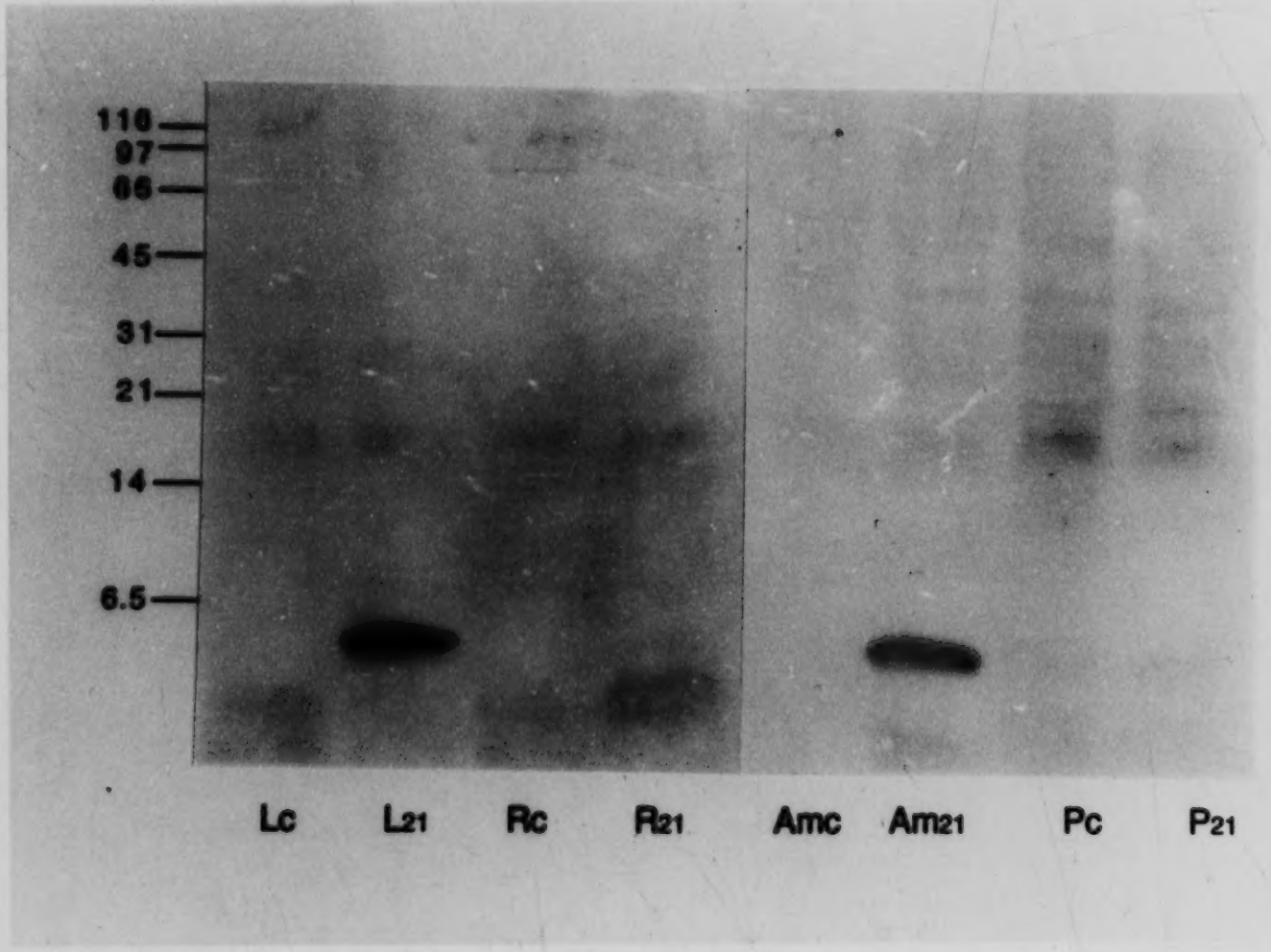

116
97
66
45
31
21
14
6.5
Lc
L21
Rc
R21
Amc
Am21
Pc
P21

Fig. 23. Western blots of SDS-soluble proteins extracted from *Brassica napus* cv. Cascade seedlings at the four leaf stage. Tissues sampled were nonacclimated leaf (Lc), 21 day acclimated leaf (La), apical meristem from 21 day acclimated plants (Am) and 21 day acclimated stem tissue both beside (S1) and further away (Sd) from the apical meristem. All lanes were loaded with 60 µg protein. Western blots were probed with anti-BN28 antibodies.

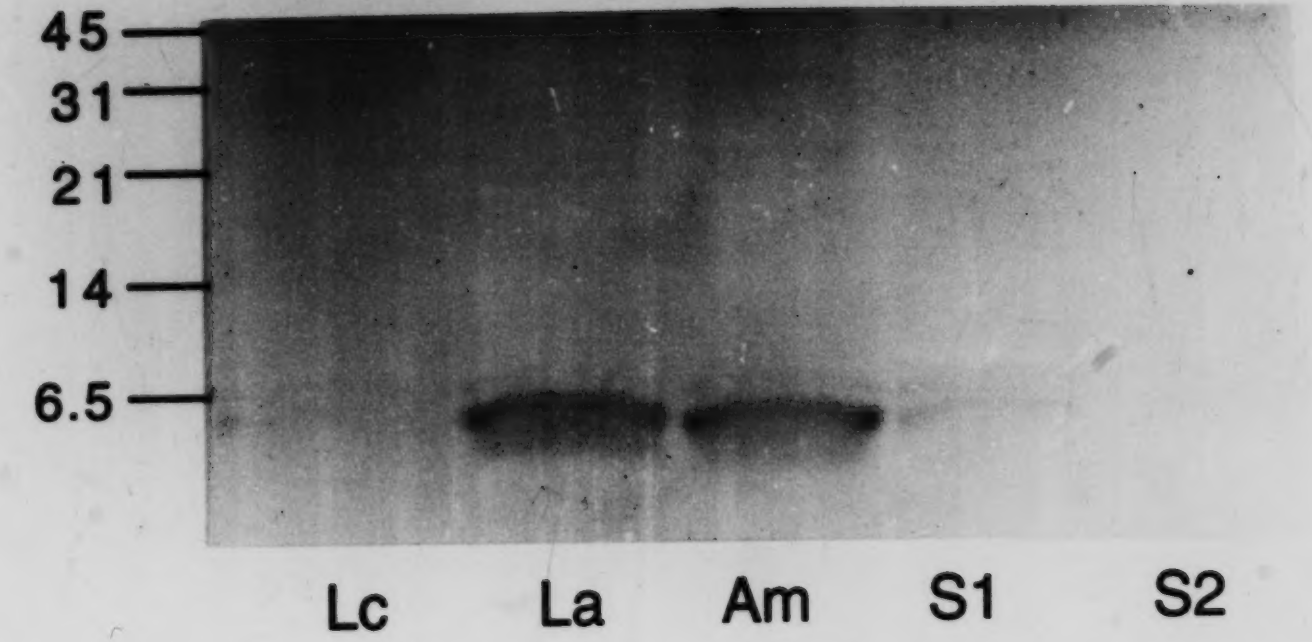

45
31
21
14
6.5
Lc
La
Am
S1
S2

Fig. 24. *Brassica napus* cv. Cascade seedlings at a) the 1 leaf stage; b) the 4 leaf stage; c) the 7 leaf stage.

a
b
c

Fig. 25. Western blot of SDS-soluble proteins extracted from 21 day acclimated *Brassica napus* cv. Cascade seedlings A) the first leaf stage; B) the four leaf stage; C) the seven leaf stage. The numbers 1 through 8 correspond to the respective emerged leaf on the seedling while Ct denotes the cotyledons. All lanes were loaded with 60 µg protein protein. Western blots were probed with anti-BN28 antibodies.

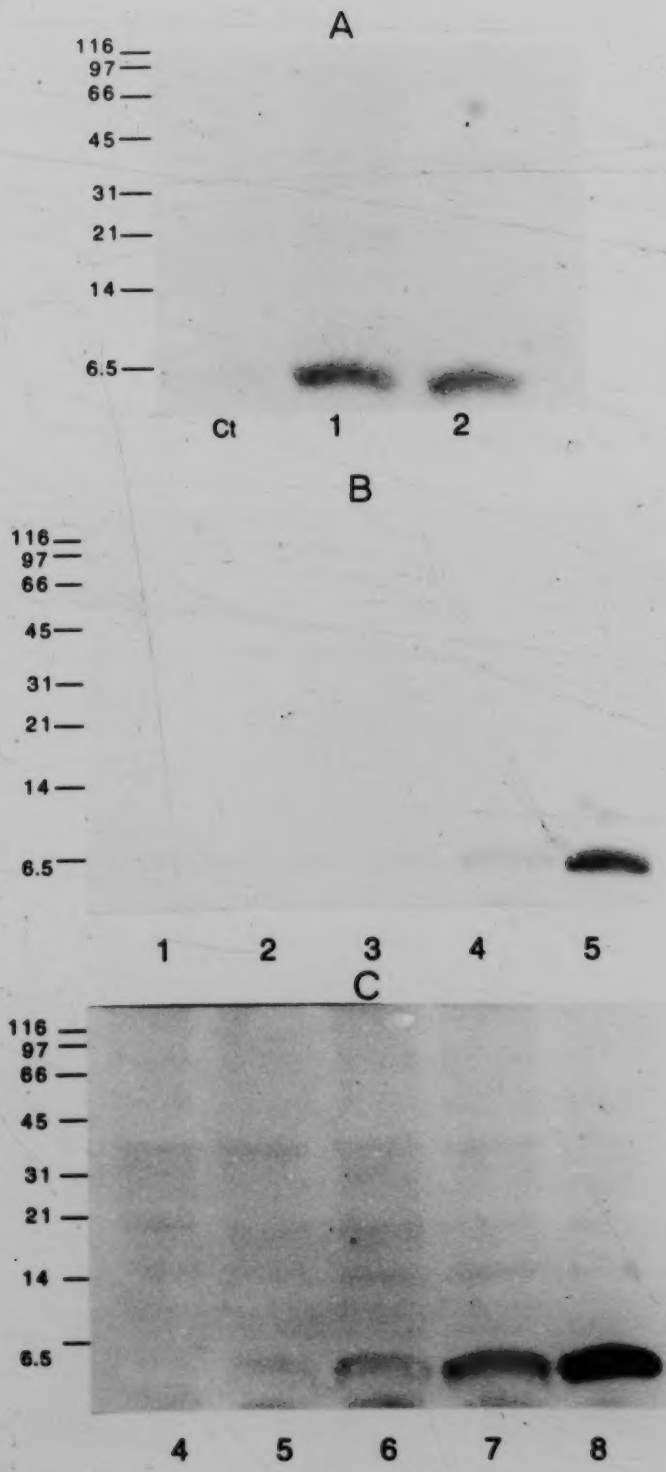

A
116
97
66
45
31
21
14
6.5
Ct
1
2
B
116
97
66
45
31
21
14
6.5
1
2
3
4
5
C
116
97
66
45
31
21
14
6.5
4
5
6
7
8

Fig. 26. Electrolyte leakage data for leaves one and four of control and 8 day acclimation *Brassica napus* cv. Cascade seedlings at the four leaf stage. (Circles represent leaf 1 sample; Triangles represent leaf 4 samples; open symbols represent 0 days acclimated samples; Filled symbols represent 8 day acclimated samples). Analysis of variance found the two leaves significantly different over the temperature range of -5°C to -10°C. ($p = 0.05$). Values represent the means of three replicates.

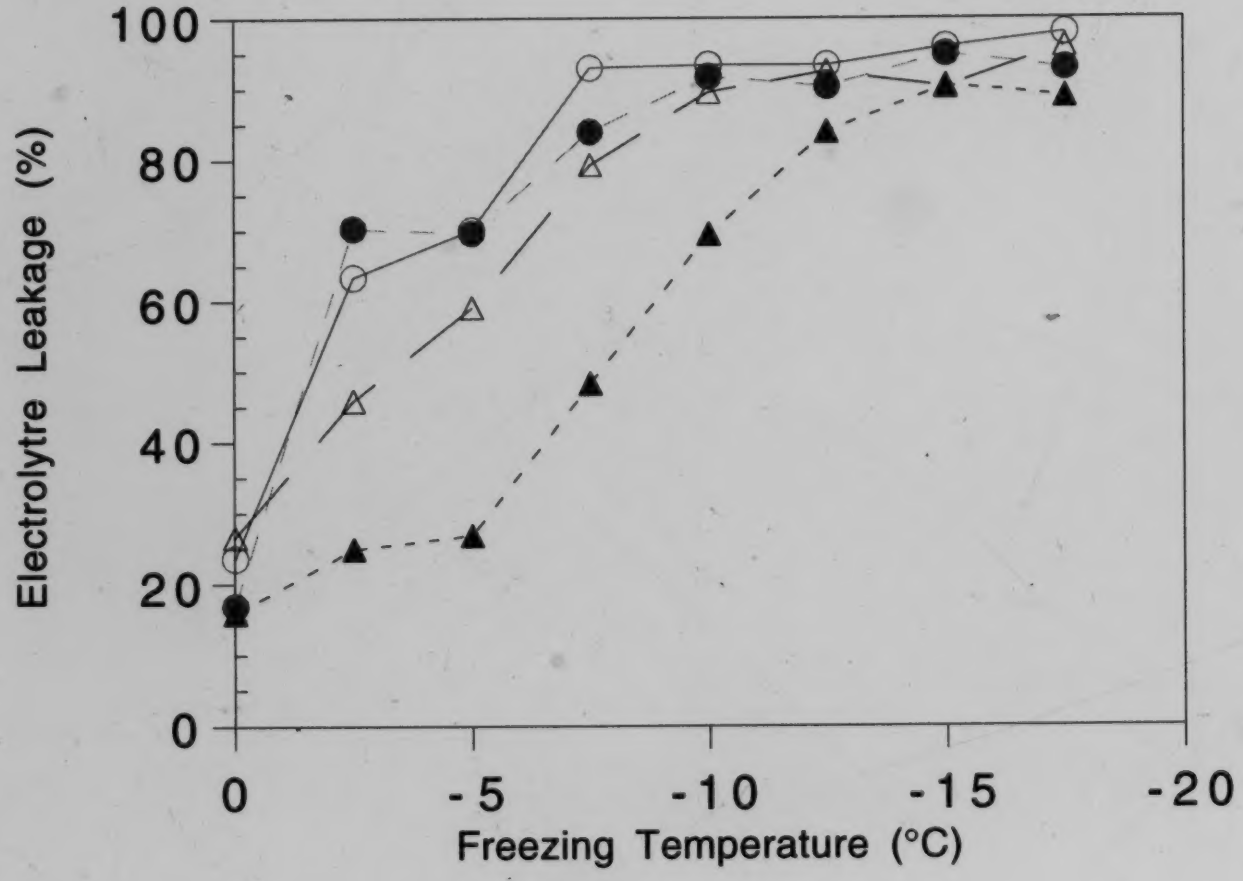

Electrolytre Leakage (%)
100
80
60
40
20
0
0
-5
-10
-15
-20
Freezing Temperature (°C)

Fig. 27. Northern hybridization of total RNA from leaves 1 and 4 of control (C) and 1, 4, and 8 day acclimated *Brassica napus* cv. Cascade seedlings at the four leaf stage. RNA loads were the same in all lanes (20 µg total RNA). The arrow indicates the 0.5kB transcript of *bn*28.

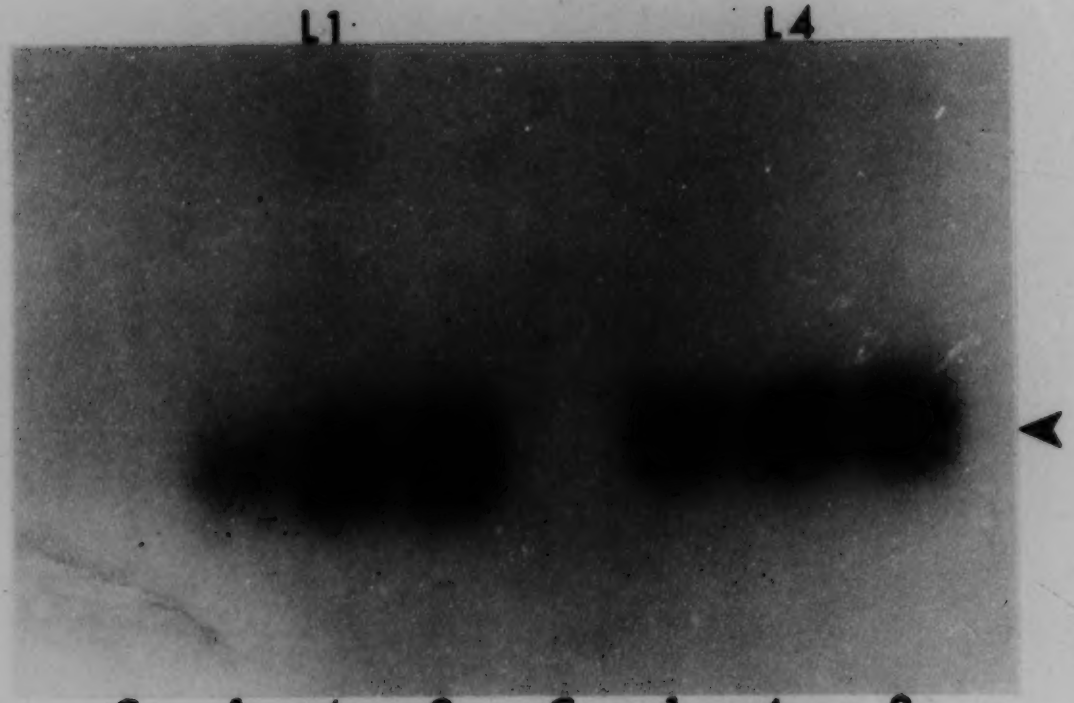

L1
L4
C 1 4 8 C 1 4 8
days at 4°C
days at 4°C

Fig. 28. Chlorophyll a/b ratios for leaves one through four of 21 day acclimated *Brassica napus* cv. Cascade seedlings at the four leaf stage. Analysis of variance found no significant difference between leaves one through four (p=0.05) indicated by the letter a above each column.

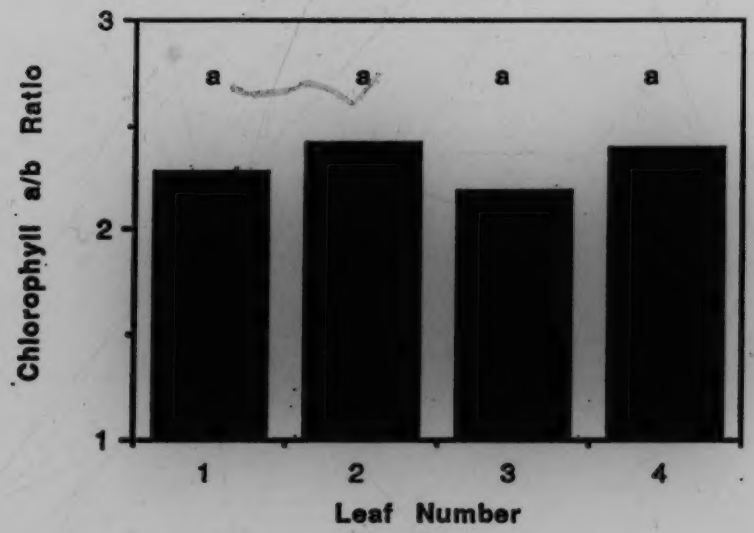

Chlorophyll a/b Ratio
3
2
1
a
a
a
a
1
2
3
4
Leaf Number

Fig. 29. Immunohistochemical controls showing reaction of purified immune and purified pre-immune serum in control and 21 day acclimated leaf tissue from *Brassica napus* cv. Cascade seedlings at the four leaf stage. Fig. A is acclimated leaf tissue tested with purified immune serum. Fig. B is acclimated leaf tissue tested with purified pre-immune serum. Figs. C is nonacclimated leaf tissue tested with purified immune serum. Fig. D is non-acclimated leaf tissue tested with purified pre-immune serum. All figures are 250 x magnification.

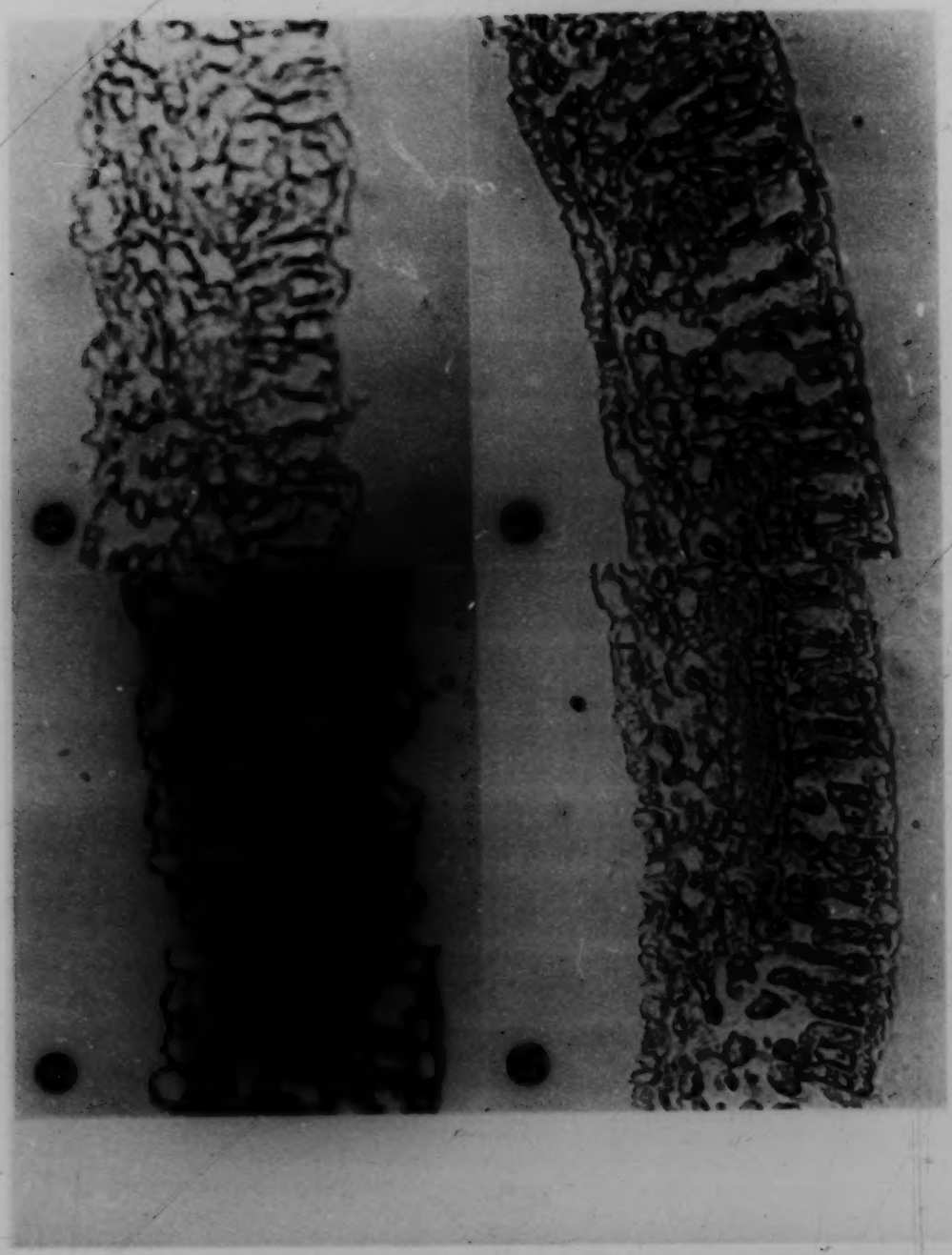


Fig. 30. Immunohistochemical localization of BN28 in control and 21 day acclimated leaf and apical meristem tissue from *Brassica napus* cv. Cascade seedlings at the four leaf stage. Fig. A is nonacclimated leaf tissue. Fig. B is acclimated leaf tissue. Figs. C is nonacclimated apical meristem tissue. Fig. D is acclimated apical meristem tissue tested with purified immune serum. All samples were probed with purified anti-BN28 polyclonal antibodies. Arrows in Fig. B indicate guard cells. Arrow in Fig. D indicates epidermal layer of young emerged leaf. Scale bar = 100 µm

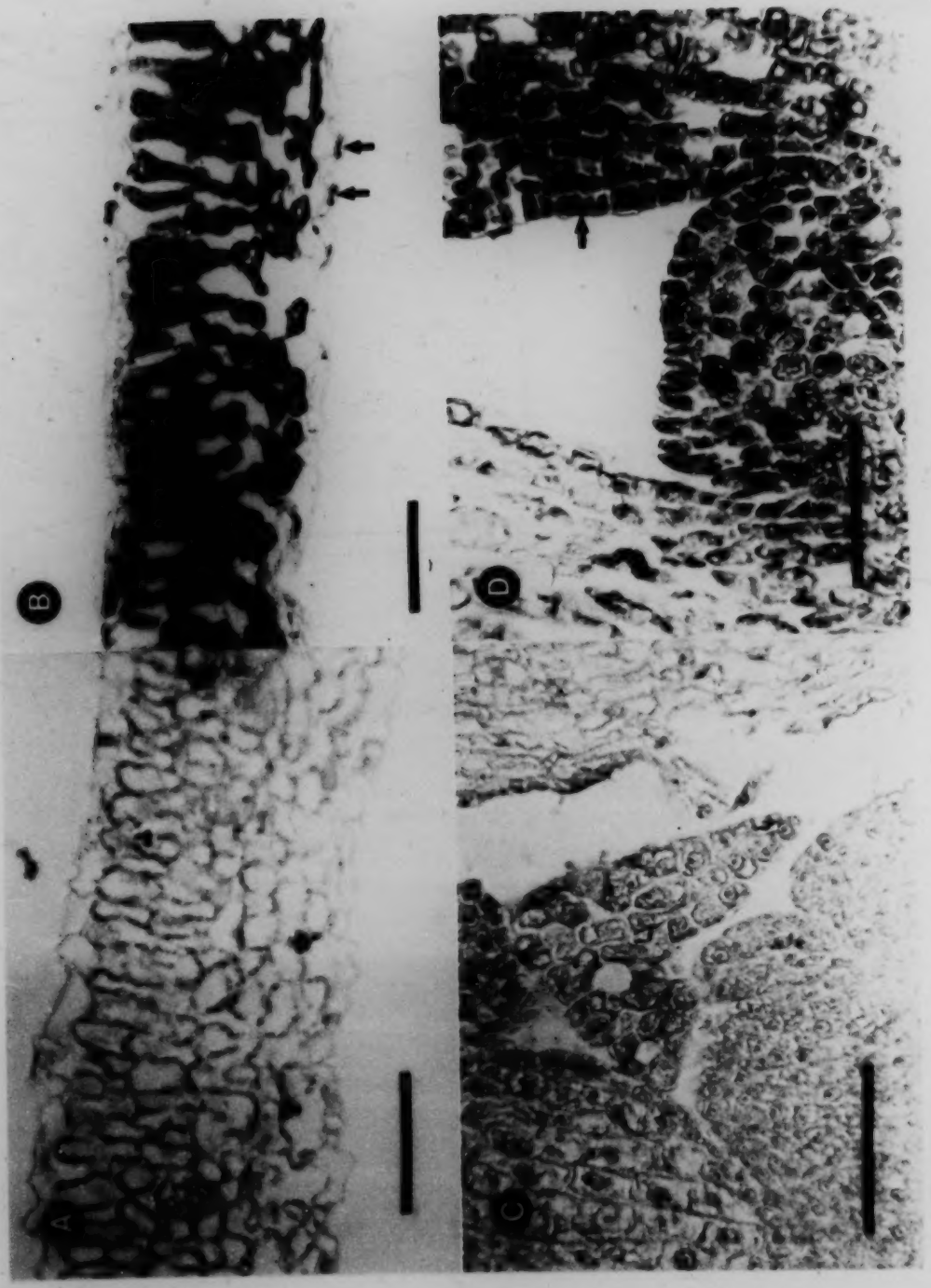

B
D
A
C

Fig. 31 Western blot of proteins from 42 day acclimated *Brassica napus* cv. Cascade seedlings at the four leaf stage. Proteins were separated by differential centrifugation at 9000 x g or 100000 x g following extraction in either high (HS) and low salt (LS) buffers. Then the pellet (p), soluble (s) fraction and total leaf protein (T) were run on an SDS-PAGE gel with 60 µg protein per lane. Western blots were probed with anti-BN28 antibodies.

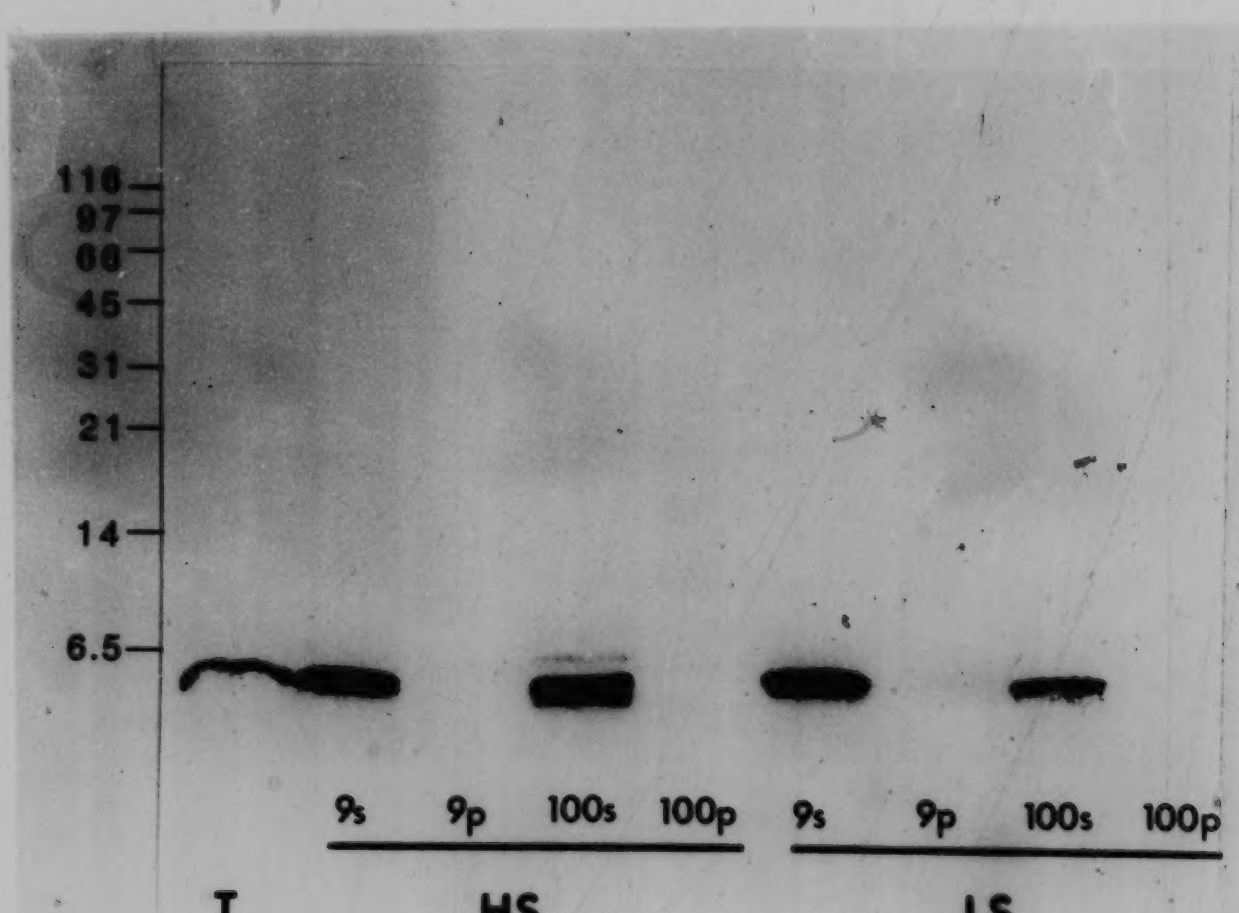

116
97
66
45
31
21
14
6.5
9s
9p
100s
100p
9s
9p
100s
100p
T
HS
LS

Fig. 32. Transmission electron micrographs of nonacclimated (Fig. A), and 21 day acclimated leaf 4 (Fig. B-D) showing immunolocalization of BN28 in *Brassica napus* cv. Cascade seedlings at the four leaf stage. All samples were probed with purified anti-BN28 polyclonal antibodies. Scale bar = 5μm. C, chloroplast; M, mitochondria; Cw, cell wall.

A
B
C
D

Fig. 33. Western blot of SDS-soluble protein from leaf 4 of four leaf stage *Brassica napus* cv. Cascade seedlings acclimated 42 days. Leaves were frozen (F) at -7.5°C for 0 to 9 hours (0, 3, 6, 9) and allowed to recover (R) at 4°C for 4 and 12 hours. All lanes were loaded with 60 µg protein. Western blots were probed with anti-BN28 antibodies.

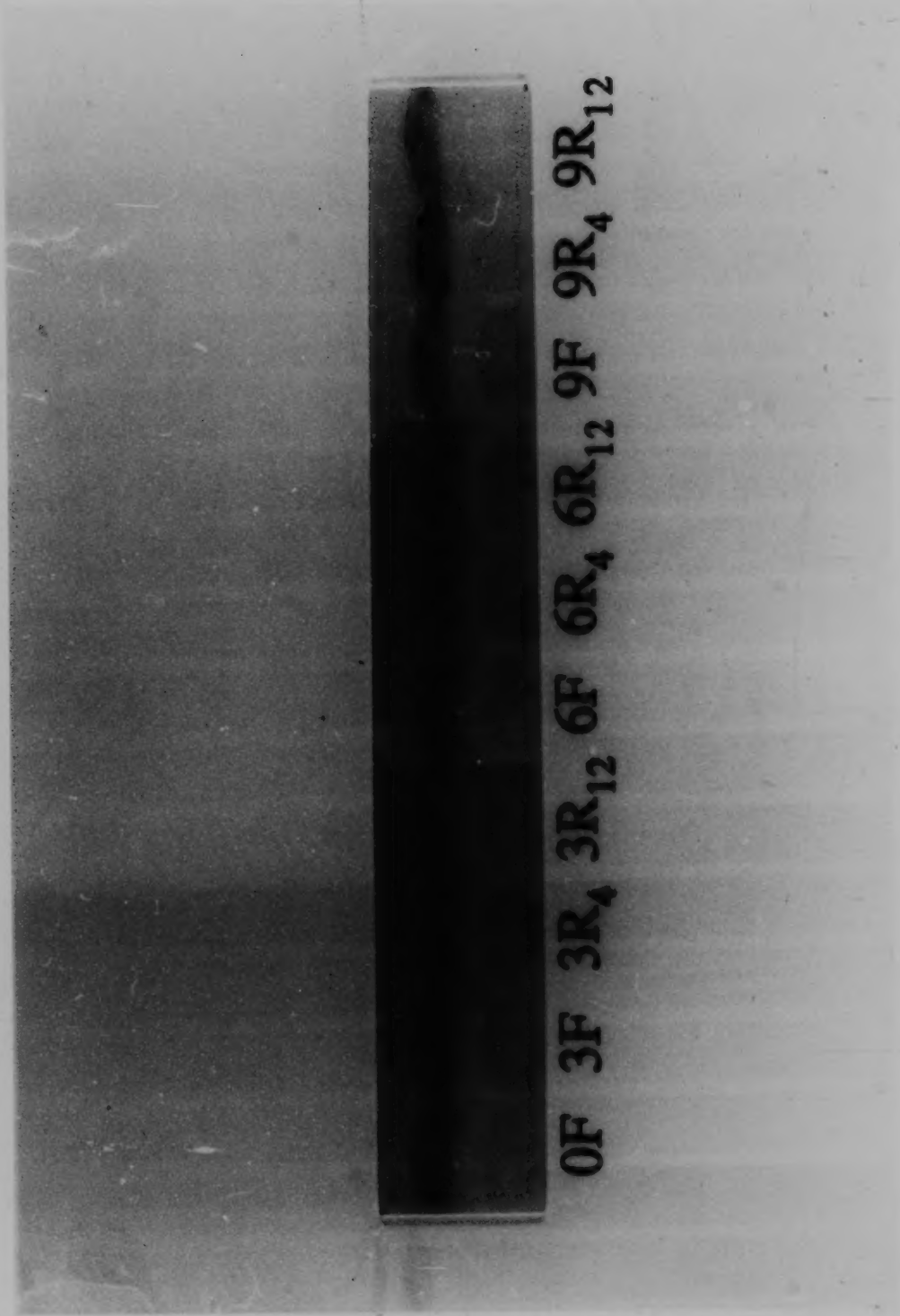

0F 3F 3R4 3R12 6F 6R4 6R12 9F 9R4 9R12

Fig. 34. Western blot of SDS-soluble proteins from leaf 4 of *Brassica napus* cv. Cascade seedlings at the 4 leaf stage. Leaf samples were from seedlings that were non-acclimated (NA), acclimated for 14 days (A), or exposed to drought stress, salt stressed or treated with 100 μM abscisic acid (mixed isomers) for 14 days. All lanes were loaded with 60 μg SDS-soluble protein. Molecular weights are shown (kDa). Western blots were probed with anti-BN28 antibodies.

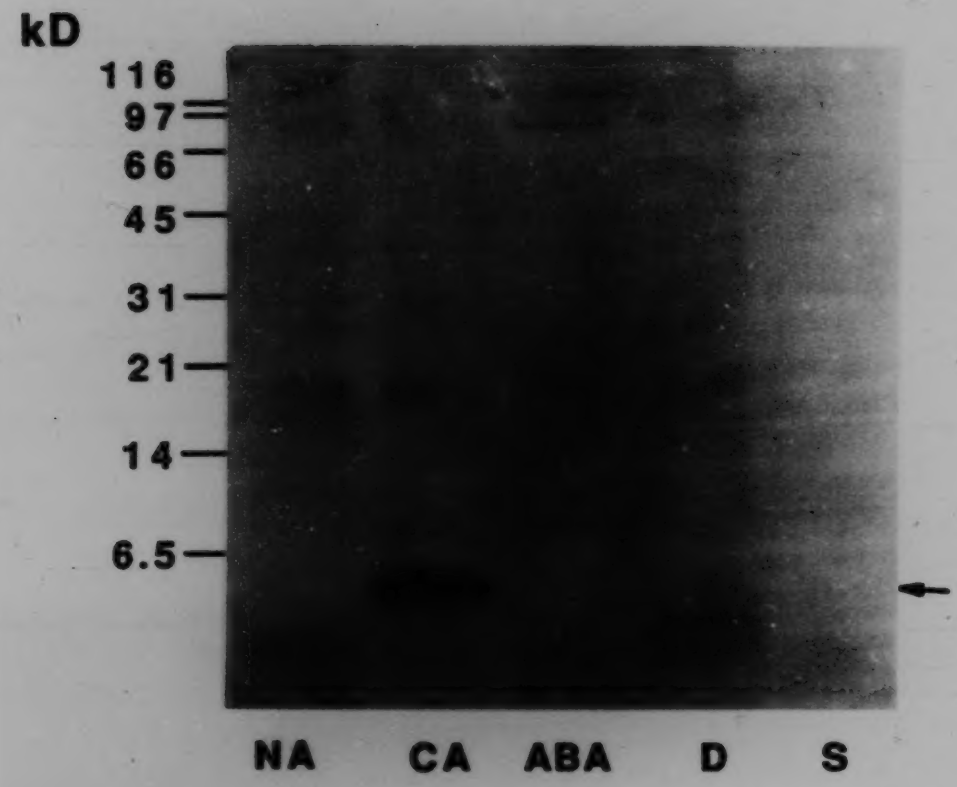

kD
116
97
66
45
31
21
14
6.5
NA
CA
ABA
D
S

Table 1 . Physiological and biochemical comparisons of BN28 from *Brassica napus*, COR6.6 and KIN1 from *Arabidopsis thaliana*.

	Brassica napus BN28	*Arabidopsis thaliana* COR6.6	*Arabidopsis thaliana* KIN1
Molecular Weight (D)	6422	6403	6500 (Predicted)
Predicted Pi	9.0	9.9	undetermined
Amino Acid Analysis	Alanine-rich	Alanine-rich	Alanine and Glycine-rich
Inducability by other stresses	Low Temp.	ABA/Drought Low Temp.	Low Temp. ABA
% Homology with BN28	100 %	69 %	66 %
Coomassie Staining	(-)	(+)	undetermined

Table 2. Purification efficiency of the ion exchange purification protocol. rBN28 was isolated from other bacterial peptides following binding of the fusion protein to DEAE cellulose and proteolytic cleavage with Factor Xa protease to release rBN28.

STEP	VOL (ml)	PROTEIN (mg)	rBN28 (mg)	PURITY (%)
CRUDE INDUCED EXTRACT	500	7500	-	-
DEAE CELLULOSE +PROTEASE TREATMENT	6.1	5.3	5.3	100

Table 3. Cryoprotective ability of rBN28, BSA, proline, and sucrose in an *in vitro* assay of LDH activity. The concentration of solute at which 50 % of the initial LDH activity is perserved (CP_{50}) is expressed as both a mass and molar value.

Compound	CP_{50} ($\mu g\ mL^{-1}$)	MW	CP_{50} (nM)
rBN28	3.2	6,420	500
BSA	12.5	66,000	200
proline	16,000	115	138,000,000
sucrose	100,000	342	292,000,000

References

Ananthanarayanan, V. S. and Hew, C.L. (1977). Structural studies on the freezing point-depressing protein of the winter flounder *Pseudopleuronecies americanus*. Biochem. Biophys. Res. Commun. 74: 685-689.

Anderson, J.V., Li, Q-B., Haskell, D.W. and Guy, C.L. (1994). Structural organization of the spinach endoplasmic reticulum-luminal 70-kilodalton heat-shock cognate gene and expression of 70-kilodalton heat-shock genes during cold acclimation. Plant Physiol. 104: 1359-1370.

Andrews, C.J. and Morrison, M.J. (1992). Freezing and ice tolerance tests for winter *Brassica*. J. Am. Sci. Agron. 84(6): 960-962.

Asahina, E. (1956). The freezing process of plant cell. Contrib. Inst. Low Temp. Sci. Hokkaido Univ. 10: 83-126.

Asghar, R., Fenton, R.D., DeMasson, D.A. and Close, T.J. (1994). Nuclear and cytoplasmic localization of maize embryo and aleurone dehydrin. Protoplasma 177: 87-94.

Back, A. and Richmond, A.E. (1971). Interrelation between GA, cytokinins and ABA in retarding leaf senescence. Physiol Plant 24: 76-79.

Bobart, J. (1684). "Physlosophical transactions and collections to the end of the year 1600" vol.2. 4th ed. pp. 155-160. Berlin, Germany.

Boothe, J.G., de Beus, M.D. and Johnson-Flanagan, A.M. (1995). Expression of a low-temperature-induced protein in *Brassica napus*. Plant Physiol. 108: 795-803.

Boothe, J.G., Sonnichsen, F.D., de Beus, M.D. and Johnson-Flanagan, A.M. (1997). Purification, characterization and structural analysis of a plant low-temperature-inducedprotein. Plant Physiol. 113: 367-376.

Brown, G.N. (1972). Changes in ribosomal patterns and a related membrane fraction during induction of cold hardiness in *Mimosa* epicotyl tissue. Plant Cell Physiol. 13: 345-351.

Burke, M.J., Bryant, R.G. and Weiser, C.J. (1974). Nuclear magnetic resonance of water in cold acclimating red osier dogwood stem. Plant Physiol. 54: 392-398.

Carpenter, J.F. and Crowe, J.H. (1988). The mechanism of cryprotection of proteins by solutes. Cryobiology 25: 244-255.

Caspary, R. (1854). Auffallende eisbildung auf pflanzen. Bot. Zeitung 12: 665-706.

Charest, C. and Phan, C.T. (1991). Kinetic properties of four enzymes involved in proline metabolism in cold-acclimated wheat. J. Exp. Botany 42 (238): 673-678.

Chauvin, L-P, Houde, M. and Sarhan, F. (1993). A leaf-specific gene stimulated by light during wheat acclimation to low temperature. Plant Mol. Biol. 23: 255-265.

Chen, P., Li, P.H. and Weiser, C.J. (1975). Induction of frost hardiness in red osier dogwood stems by water stress. Hort. Science 10: 372-374.

Chomel, M. (1710). Sur les arbres morts par la gelee de 1709 "Histoire de L'Academy Royal Des Sciences". pp.155-160.

Chou, P.Y. and Fasman, G.D. (1978). Prediction of the secondary structure of proteins from their amino acid sequence. Adv. Enzymol. Relat. Areas Mol. Biol. 47: 45-148.

Cloutier, Y. (1983). Changes in the electrophoretic patterns of the soluble proteins of winter wheat and rye following cold acclimation and desiccation stress. Plant Physiol. 71: 400-403.

Cloutier, Y. and Andrews, C.J. (1984). Efficiency of cold hardiness induction by desiccation stress in four winter cereals. Plant Physiol. 76: 595-598.

Cloutier, Y., Pelletier, L. aand Michaud, R. (1990). Development of a test for freezing in young alfalfa seedlings. Can. J. Plant Sci. 70: 307-310.

Craker, L.E., Gusta, L.V. and Weiser, C.J. (1969). Soluble proteins and cold hardiness of two woody species. Can. J. Plant Sci. 49: 279-286.

Cutler, A.J., Saleem, M., Kendall, E., Gusta, L.V., Georges, F. and Fletcher, G.L. (1989). Winter flounder antifreeze protein improves the cold hardiness of plant tissues. J. Plant Physiol. 135: 351-354.

Dallaire, S., Houde, M., Gagné, Y., Saini, H.S., Boileau, S., Chevrier, N. and Sarhan, F. (1994). ABA and low temperature induce freezing tolerance via distinct regulatory pathways in wheat. Plant Cell Physiol. 35(1): 1-9.

Daminov, J.A., Stentzler, L., Lee, S., Schwartz, J.J., Leisner, S. and Howell, S.H. (1992). Cytokinins and auxins control the expression of a gene in *Nicotiana plumbaginifolia* cells by feedback regulation. Plant Cell 4: 451-461.

Datta, N. and Cashmore, A.R. (1989). Binding of a pea nuclear protein to promoters of certain photoregulated genes is modulated by phosphorylation. The Plant Cell 1: 1069-1077.

de la Roche, I.A. (1979). Increase in linolenic acid is not a prerequisite for development of freezing tolerance in wheat. Plant Physiol. 63: 5-8.

de la Roche, I.A., Pomeroy, M.K. and Andrews, C.J. (1975). Changes in fatty acid composition in wheat cultivars of contrasting hardiness. Cryobiology 12: 506-512.

DeVries, A.L. (1971). Glycoproteins as biological antifreeze agents in antarctic fishes. Science 172: 1152-1155.

DeVries, A.L. and Lin, Y. (1977). Structure of a peptide antifreeze and mechanism of adsorption to ice. Biochim. Biophys. Acta 495: 388-392.

Dexter, S.T. (1933). Effect of several environmental factors on the hardening of plants. Plant Physiol. 8: 123-139.

Dowgert, M. F. and Steponkus, P. L. (1984). Behaviour of the plasma membrane of isolated protoplasts during a freeze-thaw cycle. Plant Physiol. 75: 1139-1151.

Duman, J.G. (1977). The role of macromolecular antifreeze in the darkling beetle *Meracantha contracta*. J. Comp. Physiol. 115: 279-286.

Duman, J.G. (1994). Purification and characterization of a thermal hysteresis protein from a plant, the bittersweet nightshade *Solanum dulcamara*. Biochim. et Biophys. Acta 1206: 129-135.

Duman, J.G., Wu, D.W., Xu, L., Tursman, D. and Olsen, T.M. (1991). Adaptations of insects to subzero temperatures. The Quarterly Review of Biology 66(4): 387-410.

Dunn, S.D. (1986). Effects of the modification of transfer buffer composition and the renaturation of proteins in gels on the recognition of proteins on Western blots by monoclonal antibodies. Anal. Biochem. 157: 144-153.

Farr, T.J., Huppe, H.C., Turpin, D.H. (1994). Coordination of chloroplastic metabolism in N-limited *Chlamydomonas reinhardtii* by redox modulation. Plant physiol. 105: 1037-1042.

Faw, W.F., Shih, S.C. and Jung, G.A. (1976). Extractant influence on the relationship between extractable proteins and cold tolerance of alfalfa. Plant Physiol. 57: 720-723.

Felix, G., Grosskopf, D.G., Regenass, M. and Boller, T. (1991). Rapid changes of protein phosphorylation are involved in transduction of the elicitor signal in plant cells. Proc. Natl. Acad. Sci. USA 88: 8831-8834.

Fickenscher, K., and Scheibe, R. (1986). Purification and properties of the cytoplasmic glucose-6-phosphate dehydrogenase from pea leaves. Arch. Biochem. Biophys. 247: 393-402.

Fowler, D.B., Limin, A.E. and Gusta, L.V. (1983). Breeding of winter hardiness in wheat. pp. 136-184. In: Eds. D.B. Fowler, L.V. Gusta, A.E. Slinkard and B.A. Hobin. New Frontiers in Winter Wheat Production. University of Saskatchewan, Saskatoon, Canada.

Gepstein, S. (1979). Photosynthesis. In: Senescence and aging in plants. pp 85-109. Academic Press Inc. New York.

Gilmour, S.J., Artus, N.N. and Thomashow, M.F. (1992). cDNA sequence analysis and expression of two cold-regulated genes of *Arabidopsis thaliana*. Plant Mol. Biol. 18: 13-21.

Gilmour, S.J., Hajela, R.K. and Thomashow, M.F. (1988). Cold acclimation in *Arabidopsis thaliana*. Plant Physiol. 87: 745-750.

Gilmour, S.J., Lin, C. and Thomashow, M.F. (1996). Purification and properties of *Arabidopsis thaliana COR* (Cold-Regulated) gene polypeptides COR15am and COR6.6 expressed in *Escherichia coli*. Plant Physiol. 111: 293-299.

Goppert, H. R. (1883). "Uber das Gefrieren, Erfrieren der Pflanzen und Schutzmittel Dagegen." pp.1-87. Enke, Stuttgart.

Gordon-Kamm, W.J. and Steponkus, P.L (1984). Lamellar-to-hexagonal$_{II}$ phase transitions in the plasma membrane of isolated protoplasts after freeze-induced dehydration. Proc. Natl. Acad. Sci. USA 81: 6373-6377.

Grant, N.H. (1969). Biochemical reactions in essentially nonaqueous systems. Cryobiology 6(3): 182-187.

Grant, N.H. and Alburn, H.E. (1967). Reactions in frozen systems VI. Ice as a possible model for biological structured-water systems. Arch. Biochem. Biophys. 118: 292-296.

Griffith, M. and Brown, G.N. (1982). Cell wall deposits in winter rye *Secale cereale* L. Puma during cold acclimation. Bot. Gaz. 143: 486-490.

Griffith, M., Ala, P., Yand, D.S.C., Hon, W-C. and Moffatt, B.A. (1992). Antifreeze protein produced endogenously in winter rye leaves. Plant Physiol. 100: 593-596.

Griffith, M., Huner, N.P.A., Espelie, K.E. and Kolattukudy, P.E. (1985). Lipid polymers accumulate in the epidermal and mestome sheath cell walls during low temperature development of winter rye leaves. Protoplasma 125: 53-64.

Guo, W., Ward, R.W., Thomashow, M.F. (1992) Characterization of a cold-regulated wheat gene related to *Arabidopsis cor*47. Plant Physiol.100: 915-922.

Gusta, L.V., Burke, M.J. and Kapoor, A.C. (1975). Determination of unfrozen water in winter cereals at subfreezing temperatures. Plant Physiol. 56: 707-709.

Guy, C.L. (1990). Cold acclimation and freezing stress tolerance: role of protein metabolism. Annu. Rev. Plant Physiol. Plant Mol. Biol. 41: 187-223.

Guy, C.L. and Haskell, D. (1987). Induction of freezing tolerance in spinach is associated with the synthesis of cold acclimation induced proteins. Plant Physiol. 84: 872-878.

Guy, C.L., Haskell, D., and Yelenoski, G. (1988). Changes in freezing tolerance and polypeptide content of spinach and citrus at 5°C. Cryobiology 25:264-271.

Guy, C.L., Huber, J.L.A. and Huber, S.C. (1992). Sucrose phosphate synthase and sucrose accumulation at low temperature. Plant Physiol. 100: 502-508.

Guy, C.L., Niemi, K.J. and Brambl, R. (1985). Altered gene expression during cold acclimation of spinach. Proc. Natl. Acad. Sci. U.S.A. 82: 3673-3677.

Hajela, R.K., Horvath, D.P., Gilmour, S.J. and Thomashow, M.F. (1990). Molecular cloning and expression of *cor* (*co*ld-*r*egulated) genes in *Arabidopsis thaliana*. Plant Physiol. 93: 1246-1252.

Hames, B.D. and Rickwood, D. (1990). Gel electrophoresis of proteins: A practical approach. pp 30-31. IRL Press London.

Harlow, E.E. and Lane, D.P. (1988). Antibodies: A laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Harris, D.A. and Bashford, C.L. (1987). Specrophotometry and spectrofluorography: A practical approach. IRL Press London.

Harvey, R.B. (1918). Hardening process in plants and development from frost injury. J. Agr. Res. 15: 83-112.

Hew, C.L. and Yang, D.S.C. (1992). Review: Protein interaction with ice. Eur. J. Biochem. 203: 33-42.

Hightower, R., Baden, C., Penzes, E., Lund, P. and Dunsmuir, P. (1991). Expression of antifreeze proteins in transgenic plants. Plant Mol. Biol. 17: 1013-1021.

Hincha, D.K., Heber, U. and Schmitt, J.M. (1989). Freezing ruptures thylakoid membranes in leaves, and rupture can be prevented *in vitro* by cryoprotective proteins. Plant Physiol 27(5): 795-801.

Hincha, D.K., Heber, U. and Schmitt, J.M. (1990). Proteins from frost-hardy leaves protect thylakoids against mechanical freeze-thaw damage *in vitro*. Planta 180: 416-419.

Holaday, A.S., Martindale, W., Alred, R., Brooks, A.L. and Leegood, R.C. (1992). Changes in activities of enzymes of carbon metabolism in leaves during exposure of plants to low temperature. Plant Physiol. 98: 1105-1114.

Hon, W-C., Griffith, M., Chong, P. and Yang, D.S.C. (1994). Extraction and isolation of antifreeze proteins from winter rye (*Secale cereale* L.) leaves. Plant Physiol. 104: 971-980.

Hon, W-C., Griffith, M., Mlynarz, A., Kwok, Y.C. and Yang, D.S.C. (1995). Antifreeze proteins in winter rye are similar to pathogenesis-related proteins. Plant Physiol. 109: 879-889.

Horwath, K.L. and Duman, J.G. (1984). Yearly variations in the over-wintering mechanisms of the cold-hardy beetle *Dendroides canadensis*. Physiol. Zool. 57: 40-45.

Houde, M., Daniel, C., Lachapelle, M., Allard, F., Laliberté, S. and Sarhan, F. (1995). Immunolocalization of freezing-tolerance-associated proteins in the cytoplasm and nucleoplasm of wheat crown tissues. Plant J. 8: 583-593.

Houde, M., Danyluk, J., Laliberte, J-F., Rassart, E., Dhindsa, R.S. and Sarhan, F. (1992). Cloning, characterization, and expression of a cDNA encoding a 50-kilodalton protein specifically induced by cold acclimation in wheat. Plant Physiol. 99: 1381-1387.

Houde, M., Danyluyk, J., Laliberté, J-F., Rassart, E., Dhinsda, R.S. and Sarhan, F. (1992). Cloning, characterization, and expression of a cDNA encoding a 50-kilodalton protein specifically induced by cold acclimation in wheat. Plant Physiol. 99: 1381-1387.

Huner, N.P.A. (1985). Morphological, anatomical, and molecular consequences of growth and development at low temperature in *Secale cereale* L. cv. Puma. Amer. J. Bot. 72(8) 1290-1306.

Huner, N.P.A. and MacDowall, F.D.H. (1976a). Chloroplastic proteins of wheat and rye grown at warm and cold-hardening temperatures. Can.J. Biochem. 54: 848-853.

Huner, N.P.A. and MacDowall, F.D.H. (1976b). Effect of cold adaptation of Puma rye on the properties of RUDP carboxylase. Biochem. Biophys. Res. Commun. 73: 411-420.

Huner, N.P.A. and MacDowall, F.D.H. (1979a). Changes in the net charge and subunit properties if ribulose bisphosphate carboxylase-oxygenase during cold hardening of Puma rye. Can. J. Biochem. 57: 15-164.

Huner, N.P.A. and MacDowall, F.D.H. (1979b). The effects of low temperature acclimation on the catalytic properties of its ribulose bisphosphate carboxylase-oxygenase. Can. J. Biochem. 57: 1036-1041.

Huner, N.P.A., Palta, J.P., Li, P.H. and Carter, J.V. (1981). Anatomical changes in leaves of Puma rye in response to growth at cold-hardening temperatures. Bot. Gaz. (Chicago) 142: 55-62.

Hurry, V.M. and Huner, N.P. (1992). Effect of cold hardening on sensitivity of winter and spring wheat leaves to short-term photoinhibition and recovery of photosynthesis. Plant Physiol. 100: 1283-1290.

Hurry, V.M., Malmberg, G., Gardeström, P. and Öquist, G. (1994). Effects of a short term shift to low temperature and of long-term cold hardening on photosynthesis and Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase and sucrose phosphate synthase activity in leaves of winter rye (*Secale cereale* L.). Plant Physiol. 106: 983-990.

Hurry, V.M., Strand, A., Tobiæson, M., Gardeström, P. and Öquist, G. (1995). Cold hardening of spring and winter wheat and rape results in differential effects on growth, carbon metabolism, and cabohydrate content. Plant Physiol 109: 697-706.

Hurry, V.M., Tobiæson, M., Krömer, S., Gardeström, P. and Öquist, G. (1995). Mitochondria contribute to increased photosynthetic capacity of leaves of winter rye (*Secale cereale* L.) following cold-hardening. Plant, Cell and Environment 18: 69-76.

Jenkins, G.I. and Woolhouse, H.W. (1991). Photosynthetic electron transport during senescence of primary leaves of *Phaseolus vulgaris*. II. The activity of photosystems one and two, and a note on the site of reduction of ferriccyanide. J. Exp. Botany 32: 989-997.

Johnson-Flanagan, A.M. and Singh, J. (1986). Membrane deletion during plasmolysis in hardened and non-hardened plant cells. Plant, Cell and Environment 9: 299-305.

Johnson-Flanagan, A.M. and Singh, J. (1987). Alteration of gene expression during the induction of freezing tolerance in *Brassica napus* suspension cultures. Plant Physiol. 85: 699-705.

Kazuoka, T. and Oeda, K. (1994). Purification and characterization of COR85-Oligomeric complex from cold-acclimated spinach. Plant Cell Physiol. 35(4): 601-611.

Kishitani, S., Watanabe, K., Yasuda, S., Arakawa, K. and Takabe, T. (1994). Accumulation of glycine betaine during cold acclimation and freezing tolerance in leaves of winter and spring barley plants. Plant, Cell and Environment 17: 89-95.

Kitaura, K. (1967). Supercooling and ice formation in mulberry trees. In "Cellular Injury and Resistance in Freezing organisms" (E. Asahina Ed.) pp. 143-156, Inst. Low Temp. Sci. Hokkaido.

Koster, K.L. and Lynch, D.V. (1992). Solute accumulation and compartmentation during the cold acclimation of Puma Rye. Plant Physiol. 98: 108-113.

Krol, M. and Huner, N.P.A. (1984). Pigment accumulation during growth and development at low temperature. In C. Sybesma (ed.) Advances in photosynthesis research, Vol IV, pp. 463-466. Martinus Nijhoff/Dr.W. Junk, The Hague.

Kurkela, S. and Borg-Franck, M. (1992). Structure and expression of *kin* 2, one of two cold- and ABA-induced genes of *Arabidopsis thaliana*. Plant Mol. Biol. 19: 689-692.

Kurkela, S. and Franck, M. (1990). Cloning and characterization of a cold- and ABA-inducible *Arabidopsis* gene. Plant Mol. Biol. 15: 137-144.

Kurkela, S., Franck, M., Heino, P., Lång, V. and Palva, E.T. (1988). Cold induced gene expression in *Arabidopsis thaliana* L. Plant Cell Reports 7: 495-498.

Kyte, J. and Doolittle, R.F. (1987). A simple method for displaying the hydropathic character of a protein. J. Mol. Biol. 157: 105-132.

LaMotte, C.E., Gochnauer, C., LaMotte, L.R., Mathur, J.R. and Davies, L.L.R. (1969). Pectin esterase in relation to leaf abscission in *Coleus* and *Phaseolus*. Plant Physiol. 44: 21-26.

Lång, V., Heino, P. and Palva, E.T. (1989). Low temperature acclimation and treatment with exogenous abscisic acid induce common polypeptides in *Arabidopsis thaliana* (L.) Heynh. Theor. Appl. Genet. 77: 729-734.

Laroche, A., Geng, X-M. and Singh, J. (1992). Differentiation of freezing tolerance and vernalization responses in Cruciferae exposed to a low temperature. Plant, Cell and Environment 15: 439-445.

Levitt, J. (1966). Cryochemistry of plant tissue. Cryobiology 3(3): 243-251.

Levitt, J. (1980). "Responses of plants to environmental stresses." vol. 2. Academic Press Inc. New York.

Lin, C. and Thomashow, M.F. (1992a). A cold-regulated *Arabidopsis* gene encodes a polypeptide having potent cryoprotective activity. Biochem. and Biophys. Research Comm. Vol 183 (3): 1103-1108.

Lin, C. and Thomashow, M.F. (1992b). DNA sequence analysis of a complementary DNA for cold-regulated *Arabidopsis* gene *cor*15 and characterization of the COR15 polypeptide. Plant Physiol. 99: 519-525.

Lin, C., Guo, W.W., Everson, E. and Thomashow, M.F. (1990). Cold acclimation in *Arabidopsis* and wheat: A response associated with expression of related genes encoding 'boiling-stable' polypeptides. Plant Physiol 94: 1078-1083.

Lund, D.B., Fennema, O. and Powrie, W.D. (1969). Enzymic and acid hydrolysis of sucrose as influenced by freezing. J. of Food Sci 34: 378-382.

Lynch, D.V. and Steponkus, P.L. (1987). Plasma membrane lipid alterations associated with cold acclimation of winter rye seedlings (*Secale cereale* L. cv. Puma). Plant Physiol. 83: 761-767.

Markert, C.L. (1963). Lactate dehydrogenase isozymes: Dissociation and recombination of subunits. Science 140:1329-1330.

Mawson, B.T. and Cummins, W.R. (1989). Thermal acclimation of photosynthetic electron transport activity by thylakoids of *Saxifraga cernua*. Plant Physiol. 89: 325-332.

Mawson, B.T. and Cummins, W.R. (1991). Low temperature acclimation of guard cell chloroplasts by the arctic plant *Saxifraga ceruna* L. Plant, Cell and Environment 14: 569-576.

Maximov, N.A. (1912). Chemische schutzmittel der pflanzen gegen erfrieren. Ber. Deutsch. Bot. Ges. 30: 52.

Mazur, P. (1960). Physical factors implicated in the death of microorganisms at subzero temperatures. Ann N.Y. Acad. Sci. 85 (Art. 2) 610-629.

McCown, B.H., Beck, G.E. and Hall, T.C. (1968). Plant leaf and stem proteins. I. Extraction and electrophoretic separation of basic, water-soluble fraction. Plant Physiol. 43: 578-582.

McCown, B.H., McLeester, R.C., Beck, G.E. and Hall, T.C. (1969). Environment-induced changes in peroxydase zymograms in the stems of deciduous and evergreen plants. Cryobiology 5: 410-412.

McKersie, B.D. and Leshem, Y.Y. (1994). Stress and stress coping in cultivated plants. Kluwer Academic Publishers, Dordrecht, Boston. London.

McKown, R.L. and Warren, G.J. (1991). Enhanced survival of yeast expressing an antifreeze gene analogue after freezing. Cryobiology 28: 474-482.

Mohapatra, S.S., Poole, R.J. and Dhindsa, R.S. (1987a). Cold acclimation, freezing resistance and protein synthesis in alfalfa (*Medicago sativa* L. cv. Saranac). Plant Physiol. 84: 1165-1172.

Mohapatra, S.S., Poole, R.J. and Dhindsa, R.S. (1987b). Changes in protein patterns and translatable messenger RNA populations during cold acclimation of alfalfa. Plant Physiol. 84: 1172-1176.

Mohapatra, S.S., Poole, R.J. and Dhindsa, R.S. (1988). Alterations in membrane protein-profile during cold treatment of alfalfa. Plant Physiol. 86: 1005-1007.

Molisch, H. (1897). "Untersuchungen uber das erfrieren der pflanzen". pp.1-23 Fisher, Jena.

Monroy, A.F., Sarhan, F. and Dhindsa, R.S. (1993). Cold-induced changes in freezing tolerance, protein phosphorylation, and gene expression: Evidence for a role of calcium. Plant Physiol. 102: 1227-1235.

Morren, C. (1838). Observations anatomiques sur la congelation des organs des vegetaux. Bull. Acad. Roy. Soc. Belles Lett. Bruxelles. 5: 65-66, 93-111.

Morrissey, I. (1983). Protein detection through silver staining. Anal. Biochem. 117: 307-309.

Murphy, L.A. and Scarth, R. (1993). Vernalization response in spring oilseed rape (*Brassica napus* L.) cultivars. Dept of Plant Sci. U of Manitoba, 927: 275-277.

Nadeau, P., Delaney, S. and Chouinard, L. (1987). Effects of cold hardening on the regulation of polyamine levels in wheat (*Triticum aestivum* L.) and alfalfa (*Medicago sativa* L.) Plant Physiol. 84: 73-77.

Neven, L.G., Haskell, D.W., Hofig, A., Li, Q-B and Guy, C.L. (1993). Characterization of a spinach gene responsive to low temperature and water stress. Plant Mol. Biol. 21: 291-305.

Öquist, G., Hurry, V.M. and Huner, N.P. (1993). Low-temperature effects on photosynthesis and correlation with freezing tolerance in spring and winter cultivars of wheat and rye. Plant Physiol. 101: 245-250.

Orr, W., Iu, B., White, T.C., Robert, L.S. and Singh, J. (1992). Complementary DNA sequence of a low temperature-induced *Brassica napus* gene with homology to the *Arabidopsis thaliana kin1* gene. Plant Physiol. 98: 1532-1534.

Ouellet, F., Houde, M. and Sarhan, F. (1993). Purification, characterization and cDNA cloning of the 200 kDa protein induced by cold acclimation in wheat. Plant Cell Physiol. 34(1): 59-65.

Paquin, R. and Pelletier, G. (1987). Influence de l'âge des plantes sur la tolérance au gel et la teneur en proline et en matière sèche de la luzerne (*Medicago media* Pers.) Oecol. Plant 22: 69-80.

Patterson, J.L. and Duman, J.G. (1978). The role of thermal hysteresis factor in *Tenenbrio molitor*. J. Exp. Biol. 74: 37-45.

Patterson, J.L. and Duman, J.G. (1982). Purification and composition of protein antifreezes with high cystein contents from larvae of the beetle *Tenebrio molitor*. J. Exp. Zool. 219: 381-384.

Pearce, R.S. and Willison, J.H.M. (1985a). A freeze-etch study of the effects of extracellular freezing on cellular membranes of wheat. Planta 163: 304-16.

Pearce, R.S. and Willison, J.H.M. (1985b). Wheat tissues freeze-etched during exposure to extracellular freezing: Distribution of ice. Planta 163: 295-303.

Peltier, G.L. (1932). A method for determination of comparative hardiness in seedling alfalfa by controlled hardening and artificial freezing. Jour. Agr. Research 44(5): 429-444

Perras, M. and Sarhan, F. (1989). Synthesis of freezing tolerance proteins in leaves, crown, and roots during cold acclimation of wheat. Plant Physiol. 89: 577-585.

Pomeroy, M.K., Siminovitch, D. and Wightman, F. (1970). Seasonal biochemical changes in the living bark and needles of red pine (*Pinus resinosa*) in relation to adaption to freezing. Can. J. Bot. 48: 953-967.

Ramsay, J.A. (1964). The rectal complex of the mealworm, *Tenebrio molitor* L. (Coleoptera, Tenebrionidae). Phil. Trans. Roy. Soc. London B. 248: 279-314.

Raymond J.A. and DeVries, A.L. (1972). Freezing behaviour of fish blood glycoproteins with antifreeze proteins. Cryobiology 9: 541-547.

Robertson, A.J., Gusta, L.V., Reaney, M.J.T. and Ishikawa, M. (1988). Identification of proteins correlated with increased freezing tolerance in Bromegrass (*Bromus inermis* Leyss. cv Manchar) cell structures. Plant Physiol. 86: 344-347.

Robson, B. and Garnier, J. (1978). Analysis of the accuracy and implications of simple methods for predicting the secondary structure of globular proteins. J. Mol. Biol. 120: 97-120.

Sakai, A. and Larcher, W. (1987). Frost survival of Plants: Responses and adaptations to freezing stress. Springer-Verlag, Berlin and New York.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989). Molecular cloning: A laboratory manual, Ed 2. Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

Santarius, K.A. (1982). The mechanism of cryoprotection of biomembrane systems by carbohydrates. In : Li PH, Sakai A. (eds) Plant cold hardiness and freezing stress, vol II. Academic Press, London, New York, pp 475-486.

Santarius, K.A. and Giersch, C. (1983). Cryopreservation of spinach chloroplast membranes by low-molecular-weight carbohydrates. II. Discrimination between colligative and non-colligative protection. Cryobiology 20: 90-99.

Santarius, K.A. and Giersch, C. (1984). Factors contributing to inactivation of isolated thylakoid membranes during freezing in the presence of variable amounts of glucose and NaCl. Biophys. J. 46: 129-139.

Sarhan, F. and Chevrier, N. (1985). Regulation of RNA synthesis by DNA-dependent RNA polymerases and RNases during cold acclimation in winter and spring wheat. Plant Physiol. 78: 250-255.

Sarhan, F. and Perras, M. (1987). Accumulation of a high molecular weight protein during cold hardening of wheat (*Triticum aestivum* L.). Plant Cell Physiol. 28: 1173-1179

Sarokin, L.P. and Chua, N-H. (1992). Binding sites for two novel phosphoproteins, 3AF5 and 3AF3, are required for *rbcS-3A* expression. Plant Cell 4: 473-483.

Scarth, G.W. (1936). The yearly cycle in the physiology of trees. Trans. Roy. Soc. Can. Sect. 5 30: 1-10.

Scarth, G.W. and Levitt, J. (1937). The frost-hardening mechanism of plant cells. Plant Physiol. 12: 51-78.

Schagger, H. and von Jagow, G. (1987). Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Anal. Biochem. 166: 368-379.

Schneppenheim, R. and Theede, H. (1980). Isolation and characterization of freezing point depression peptides from larvae of *Tenebrio molitor*. Comp. Biochem. Physiol. B. 67: 561-568.

Scholander, P.F. and Maggert, J.E. (1971). Supercooling and ice propagation in blood from arctic fish. Cryobiology 8: 371-374.

Scholander, P.F., VanDam, L., Kanwisher, J.W., Hammel H.T. and Gordon, M.S. (1957). Supercooling and osmoregulation in arctic fish. J.Cell Comp. Physiol. 49: 5-24.

Sieg, F., Schröder, W., Schmitt, J.M. and Hincha, D.K. (1996). Purification and characterization of a cryoprotective protein (Cryoprotectin) from the leaves of cold-acclimated cabbage. Plant Physiol. 111: 215-221.

Siminovitch, D. and Briggs, D.R. (1949). The chemistry of the living bark of the black locust tree in relation to frost hardiness. I. Seasonal variations in protein content. Arch. Biochem. Biophys. 23: 8 -17.

Siminovitch, D. and Cloutier, Y. (1982). Twenty-four-hour induction of freezing and drought tolerance in plumules of winter rye seedlings by desiccation stress at room tempurature. Plant Physiol. 69: 250-255.

Siminovitch, D. and Scarth, G. W. (1938). A study of the mechanism of frost injury to plants. Can. J. Res. C16: 467-481.

Siminovitch, D., Rheaume, B., Pomeroy, K. and Lepage, M. (1968). Phospholipid, protein and nucleic acid increases in protoplasm and membrane structures associated with development of extreme freezing resistance in black locust tree cells. Cryobiology 5:202-225.

Singh, J., Iu, B. and Johnson-Flanagan, A.M. (1987). Membrane alterations in winter rye and *Brassica napus* cells during lethal freezing and plasmolysis. Plant, Cell and Environment 10: 163-168.

Singh, M. (1995). Photosynthetic genes and cold acclimation. Ph.D. thesis, Department of Plant Science, University of Alberta, Edmonton, Alberta.

Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J. and Klenk, D.C. (1985). Measurement of protein using bicinchoninic acid. Anal. Biochem. 150: 76-85.

Sonnichsen, F.D., Van Eyk, J.E., Hodges, R.S., and Sykes, B.D. (1992). Effect of trifluoroethanol on protein structure: An NMR and CD study using a synthetic actin peptide. Biochemistry 31: 8790-8797.

Steponkus, P.L. (1984). Role of the plasma membrane in freeze injury and cold acclimation. Annu. Rev. Plant Plysiol. 35: 543-584.

Steponkus, P.L., Uemura, M., Balsamo, R.A., Arvinte, T. and Lynch, D.V. (1988). Transformation of the cryobehaviour of rye protoplasts by modification of the plasma membrane lipid composition. Proc. Natl. Acad. Sci. USA 85: 9026-9030.

Stone, J.M., Palta, J.P., Bamberg, J.B., Wiess, L.S. and Harbage, J.F. (1993). Inheritance of freezing resistance in tuber-bearing *Solanum* species: Evidence for independent genetic control of non-acclimated freezing tolerance and cold acclimation capacity. Proc. Natl. Acad. Sci. USA Vol 90: 7869-7873.

Sukumaran, N.P. and Weiser, C.J. (1972). An excised leaflet test for evaluating potato frost tolerance. Hortic. Sci. 7: 467-468.

Tamiya,T., Okahashi, N., Sakuma, R., Aoyama, T., Akahane, T. and Matsumoto, J.J. (1985). Freeze denaturation of enzymes and its prevention with additives. Cryobiology 22:446-456.

Thomashow, M.F. (1990). Molecular genetics of cold acclimation in higher plants. Advances in Genetics Vol 28: 99-131.

Tong, M-M. and Pincock, R.E. (1969). Denaturation and reactivity of invertase in frozen solutions. Biochemistry 8(3): 908-913.

Tronsmo, A.M. (1984). Predisposing effects of low temperature on resistance to winter stress factors in grasses. Acta Agric. Scand 34: 210-220.

Tronsmo, A.M. (1985) Induced resistance to biotic stress factors in grasses by frost hardening. *In* Å Kaurin, O Junttila , J Nilsen, eds. Plant Production in the North. Norwegian University Press, Tromso. Norway, pp 127-133.

Tronsmo, A.M. (1993). Resistance to winter stress factors in half-sib families of *Dactylis glomerata*, tested in a controlled environment. Acta Agric. Scan 43: 89-96.

Trunova, T.I. (1982). Mechanism of winter wheat hardening at low temperature. In Plant cold hardiness and freezing stress: Mechanisms and crop implications. (P.H. Li and A. Sakai, eds) 2: 41-54. Academic Press, New York.

Uemura, M. and Steponkus, P.L. (1989). Effect of cold acclimation on the incidence of two forms of freezing injury in protoplasts isolated from rye leaves. Plant Physiol 91: 1131-1137.

Uemura, M. and Steponkus. P.L. (1994). A contrast of the plasma membrane lipid composition of oat and rye leaves in relation to freezing tolerance. Plant Physiol. 104: 479-496.

Uemura, M. and Yoshida, S. (1984). Involvement of plasma membrane alterations in cold acclimation of winter rye seedlings (*Secale cereale* L. cv Puma). Plant Physiol. 75: 818-826.

Uemura, M. Gilmour, S.J., Thomashow, M.F. and Steponkus, P.L. (1996). Effects of COR6.6 and COR15am polypeptides encoded by *COR* (Cold-Regulated) genes of *Arabidopsis thaliana* on the freeze-induced fusion and leakage of liposomes. Plant Physiol. 111: 313-327.

Urrutia, M.E., Duman, J.G. and Knight, C.A. (1992). Plant thermal hysteresis proteins. Biochim. Biophys. Acta 1121: 199-206.

Vernon, L.P. (1960). Spectrophotometric determination of chlorophylls and pheophytins in plant extract. Anal. Chem. 32(9): 1144-1150.

Volger, H.G. and Heber, U. (1975). Cryoprotective leaf proteins. Biochim. Biophys. Acta 412: 335-349.

Webb, M.S. and Steponkus, P.L. (1992). Ultrastructural manifestations of freezing injury in protoplasts isolated from cold-acclimated and non-acclimated oat leaves. Cryobiology 29: 710-711.

Webb, M.S. and Steponkus, P.L. (1993). Freeze-induced membrane ultrastuctural alterations in rye (*Secale cereale*) leaves. Plant Physiol. 101: 995-963.

Webb, M.S., Gilmour, S.J., Thomashow, M.J. and Steponkus, P.L. (1996). Effects of COR6.6 and COR15am polypeptides encoded by *COR* (Cold-Regulated) genes of *Arabidopsis thaliana* on dehydration-induced phase transitions of phospholipid membranes. Plant Physiol. 111: 301-312.

Webb, M.S., Uemura, M. and Steponkus, P.L. (1994). A comparison of freezing injury in oat and rye: Two cereals at the extremes of freezing tolerance. Plant Physiol. 104: 467-478.

Weiser, C.J. (1970). Cold resistance and injury in woody plants. Science 169: 1269-1278.

Wen, D. and Laursen, R.A. (1992). Structure-function relationships in an antifreeze polypeptide: The role of neutral, polar amino acids. J. Biol. Chem. 267(20): 14102-14108.

Weretilnyk, E., Orr, W., White, T.C., Iu, B. and Singh, J. (1993). Charaterization of three related low-temperature-regulated cDNAs from winter *Brassica napus*. Plant Physiol. 101: 171-177.

White, T.C., Simmonds, D., Donaldson, P. and Singh, J. (1994). Regulation of BN115, a low temperature responsive gene from winter *Brassica napus*. Plant. Physiol. 106: 917-928.

Wilhelm, K.S. and Thomashow, M.F. (1993). *Arabidopsis thaliana cor* 15b, an apparent homologue of *cor* 15a, is strongly responsive to cold and ABA, but not drought. Plant Mol. Biol. 23: 1073-1077.

Withers, L.A. and King, P.J. (1979). Proline: A novel cryoprotectant for the freeze preservation of cultured cells of *Zea mays* L. Plant Physiol. 64: 675-768.

Wu, D.W., Duman, J.G., Cheng, C-H.C. and Castellino, F.J. (1991). Purification and charaterization of antifreeze proteins from larvae of the beetle *Dendroides canadensis*. J. Comp. Physiol. B 161: 271-278.

Zachariassen, K.L. (1985). Physiology of cold tolerance in insects. Physiol. Rev. In Amer. Physiol. Soc. Vol 65 (4): 799-832.

Zettle, J. (1975). Cold hardiness strategies and thermal hysteresis in Collembola. Rev. Ecol. Biol. Sol. 21: 189-203.

Appendix

Accumulation of BN28 during cold acclimation (CA) in *Brassica napus* leaves. (µg and pmol BN28 mg^{-1} protein)

CA	µg BN28[a]	pmol BN28[a]
d		
8	0.414 ± 0.096	63.2
14	0.396 ± 0.040	60.5
28	0.542 ± 0.014	82.7
42	0.494 ± 0.026	75.4

[a] µg and pmol BN28 mg^{-1} protein.

(Boothe et al. 1995)